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Production, Properties and Utility of Bacterial Minicells

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With 8 Figures

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I. Introduction

Minicells are small non-growing bodies produced by aberrant cell divisions at the polar ends of rod-shaped bacteria; as such, they are anucleate or DNA-deficient and approximately spherical in shape. Minicells are produced either rarely in wild-type strains of bacteria or continuously during growth in mutant strains of bacteria. HOFFMAN and FRANK (1963) carefully documented one instance of the rare production of a minicell from one cell in a growing culture of *Escherichia coli*. The first report of an apparent minicell-producing strain was made by GARDNER (1930) who described an unequal cell division process in a strain of *Vibrio cholera* that resulted in the production of spherical non-growing granules and he discussed the occurrence of similar aberrant cell divisions among "bacilli of the typhoid-coli and other groups". The formation of apparent minicells by strains of *Spirillum serpens* has also been described (PEASE, 1956), and TANAMI and YAMADA (1973) have recently reported the apparent production of minicell-like bodies by the obligate intracellular parasite, *Chlamydia psittaci*.

VOROS and GOODMAN (1965) isolated a minicell-producing strain of *Erwinia amylovora* and described some of its distinctive growth and minicell-forming properties. ADLER et al. (1966, 1967) characterized a minicell-producing mutant strain of *E. coli* in some detail, introduced the term "minicell", and underlined the potential usefulness of minicells and minicell-producing mutants. Subsequently, many minicell-producing strains have been isolated in gram-negative and gram-positive bacteria, including *Salmonella typhimurium* (EPPS and IDZIAK, 1970; TANKERSLEY, 1970; TANKERSLEY and WOODWARD, 1973), other *Salmonella* species including *S. anatum*, *S. enteritidis*, *S. pullorum*, *S. senftenberg*, and *S. worthington* (EPPS and IDZIAK, 1970), the marine pseudomonad strain B-16 (FORSBERG et al., 1970a, b), *Bacillus subtilis* (VAN ALSTYNE and SIMON, 1971; REEVE et al., 1973), and *Haemophilus influenzae* (SETLOW et al., 1973).

Minicells are produced throughout the growth cycle of minicell-producing mutant strains and contain RNA and protein but little or no chromosomal DNA. Hence, they have been very useful in studies on cell division, the localization of enzymes and constituents in cells, transport processes, the synthesis and function of cell wall and membrane, infection by bacteriophages, and the mechanism of bacterial conjugation. The more recent discovery that plasmid DNA in plasmid-containing minicell-producing strains segregates into minicells of *E. coli* (Inselburg, 1970; Kass and Yarmolinsky, 1970; Levy and Norman, 1970; Roozen et al., 1971 a) and *S. typhimurium* (Sheehy et al., 1973 a, b) has provided a model system for studies on DNA replication, recombination, repair of radiation-induced damage, and transcription and translation of plasmid-specified genetic information. The fact that plasmid-containing minicells can act as genetic donors during conjugation (Kass and Yarmolinsky, 1970; Levy and Norman, 1970; Roozen et al., 1971 a) and can be productively infected with bacterial viruses (Roozen et al., 1971 b; Curtis, unpubl.) has also contributed to the potential usefulness of the minicell system.

This review attempts to summarize all available information, both published and unpublished, on minicells and minicell-producing strains. We will not, however, discuss those temperature-sensitive, division-defective mutants of *E. coli* and *B. subtilis* that only produce DNA-deficient cells at temperatures restrictive for normal cell division. The reader interested in these mutants is referred to the review by Hirota and Ricard (1972). Since minicell research is a rather new field of study, we also include a discussion of methods (Appendices I and III) and available strains (Appendix II, Tables 3 and 4) that will hopefully benefit those interested in using the minicell system. An earlier review of the minicell-producing strains of *E. coli* has been published (Adler and Hardigree, 1972).

II. General Properties of Minicell-Producing Strains

A. Relationship of Minicell Formation to Growth and Normal Cell Division

Minicells are produced throughout the growth cycle, at all temperatures at which growth occurs and in all types of liquid and solid media by the minicell-producing mutants of *E. coli* (Adler et al., 1967; Adler and Hardigree, 1972), *S. typhimurium* (Sheehy, unpubl.) and *B. subtilis* (Reeve et al., 1973). The growth phase of a culture as well as the growth medium can influence minicell production. For example, minicell formation is most frequent in *B. subtilis* strains in the late-log to early-stationary phase when the frequency of division events increases and shorter cells rather than filaments come to predominate in cultures (Reeve et al., 1973; Mendelson and Reeve, 1973). Khachatourians (pers. comm., 1973) has observed that the doubling time for minicell number can be less than the doubling time for cell number in an *E. coli* minicell-producing strain, and that minicells continue to be produced and to increase in

number for as long as 7 hours into stationary phase. We have observed that the yield of minicells produced by *E. coli* and *S. typhimurium* strains varies with the growth medium such that higher yields are observed on media that allow faster growth rates. For instance: 3XD medium (FRASER and JERRELL, 1953) and minimal liquid medium (CURTISS, 1965) supplemented with 1.5% Casamino acids and 3.0% glycerol (or 0.5% glucose) give the highest yields; Penassay, L broth (LENNOX, 1955), and minimal liquid medium containing 0.5% glucose with only 0.5% Casamino acids give high yields; minimal liquid medium containing 0.5% glucose with no Casamino acids gives low yields, and even lower yields are obtained with succinate or acetate as carbon sources. VOROS and GOODMAN (1965) observed that increased osmolarity of the growth medium decreased both the amount of minicell production and the length of filaments in a minicell-producing filamentous strain of *E. amylovora*.

Since minicell formation constitutes an aberration in cell division, the mechanism of minicell-production is of considerable interest. The process of septum formation has been compared for normal divisions and minicell-producing division by examining septum ultrastructure and by studying the growth patterns of minicell-producing strains under a variety of experimental conditions. Electron micrographs of thin sections of minicell-producing strains of *E. coli* (Fig. 1), *B. subtilis* (VAN ALSTYNE and SIMON, 1971; REEVE et al., 1973, and Fig. 2A–D) and *E. amylovora* (HUANG and GOODMAN, 1970) reveal that the ultrastructure of a septum between a minicell and parent cell is the same as that of a septum between two progeny cells undergoing normal cell division. REEVE et al. (1973) have also demonstrated (Fig. 2B, C and E) that mesosomes are often apparent in thin sections of *B. subtilis* minicells. This supports the idea that mesosomes are important in septum formation and that mesosomes are not always associated with chromosomal DNA. NAUMAN et al. (1971) reported that ribosomal helices, which appear intracellularly as a result of growth under increasingly acidic conditions, formed in the division plane of minicells and cells of the *E. coli* strain P678–54. KHACHATOURIANS (pers. comm., 1973) has observed that septum formation in *E. coli* takes the same amount of time regardless of whether the site of septum formation is central or at the polar region of the cell. The diameter of minicells is often smaller than the diameter of parental rod-shaped cells in many minicell-producing systems including *E. coli* (FRAZER and CURTISS, 1973), *E. amylovora* (VOROS and GOODMAN, 1965; HUANG and GOODMAN, 1970), the marine pseudomonad of FORSBERG et al. (1970b), *S. typhimurium* (CURTISS and SHEEHY, unpubl. observations) and *B. subtilis* (VAN ALSTYNE and SIMON, 1971; REEVE et al., 1973). Hence, the site of a minicell-forming septum although variable may often be quite close to the cell pole.

It is clear from studies of growth patterns that minicell formation occurs only under conditions that permit normal cell division and chromosome replication. ADLER et al. (1969) demonstrated that if a *lon*$^-$ mutation (i.e. a mutation causing cell division, but not cell growth to be blocked following exposure of cells to radiation) was present in an *E. coli* minicell-producing strain, minicell

Fig. 1. Thin section of an *E. coli* minicell-producing cell (χ925) dividing to yield a minicell. ×49140. Electron micrograph taken by D. P. ALLISON

formation was inhibited as effectively as cell division following radiation. CLARK (1968a, b) showed that when an *E. coli* minicell-producing strain was incubated in the presence of nalidixic acid (which inhibits DNA synthesis and thereby also blocks cell division), no minicells were formed. REEVE (pers. comm., 1971) and KHACHATOURIANS et al. (1973) demonstrated that if the *bug6*$^-$ thermosensitive cell division mutation described by REEVE et al. (1970) (i.e. a mutation causing formation of filaments at the non-permissive temperature that subsequently undergo a burst of cell division when reincubated at the permissive temperature) was present in a minicell-producing strain, no minicells were formed at the non-permissive temperature. Other studies (KHACHATOURIANS, pers. comm., 1973) have demonstrated that both minicell formation and normal cell division is blocked when cultures are exposed to a variety of treatments that interfere with either chromosome replication or septum formation (e.g. presence of

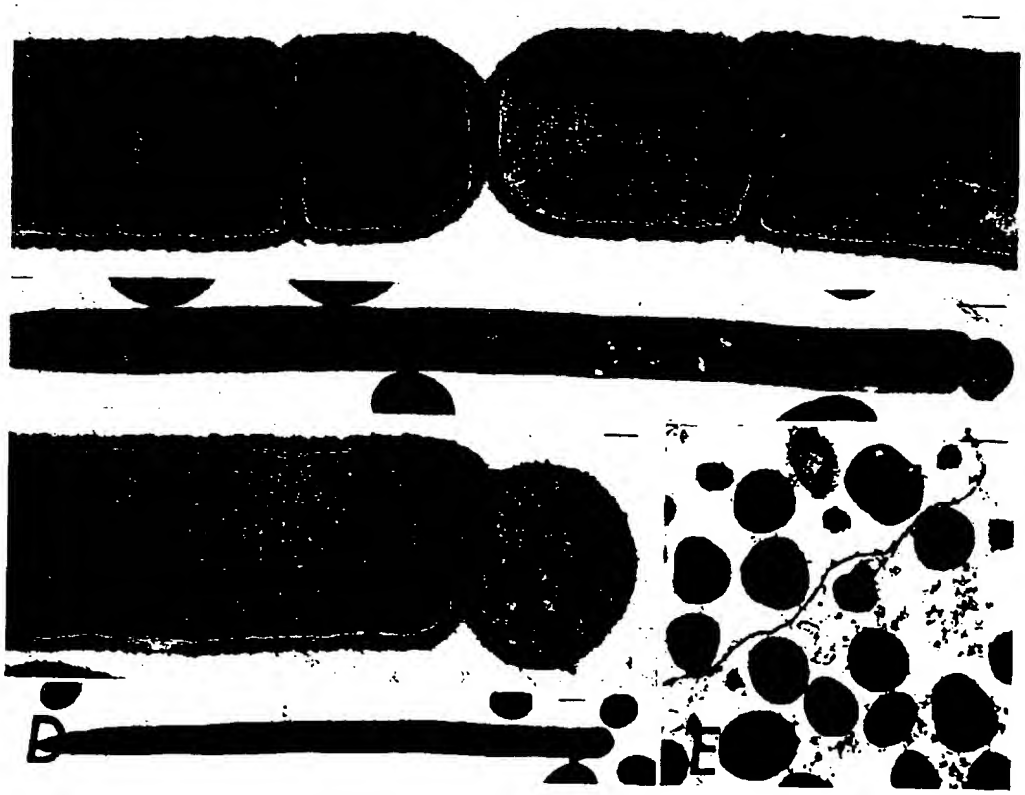

Fig. 2A—E. Thin sections of *B. subtilis divIV-B1* (strain CU 403). A Adjacent minicells formed from sister poles. ×41400. B Terminal minicell production by filamentous cell. ×12600. C Same cell and minicell as in B demonstrating mesosome attached to minicell septum. ×41400. D Multiple minicell production. ×5400. E Purified minicells. ×12600. From REEVE et al. (1973) with permission of the authors and American Society for Microbiology

chloramphenicol, thymine starvation, amino acid starvation, delay of division following UV-irradiation, and nutritional shift-up experiments in which a culture is shifted from a minimal to complex medium) and that in many instances, when inhibition of cell division is released, minicell formation precedes reinitiation of normal cell division. All these studies suggest that the septation process resulting in the formation of minicells is normal in all respects except for the placement of the septum (ADLER and HARDIGREE, 1972; REEVE et al., 1973).

A number of investigators have followed the growth patterns of individual clones of minicell-producing strains for many generations in order to precisely describe the process of septum placement. The results of these studies with *E. coli* strains indicate that a cell cannot simultaneously form a minicell septum and a centrally placed septum, suggesting that cells are restricted in their septum-forming capacity (ADLER et al., 1969; ADLER and HARDIGREE, 1972; TEATHER et al., 1974). REEVE (pers. comm., 1971) and KHACHATOURIANS et al. (1973), studying a minicell-producing *bug6*$^-$ mutant, also noted that *E. coli* cells possess a limited capacity for the total number of septa that can form

during the burst of division that occurs when filaments formed at 42° C are shifted down to a temperature permissive for septum formation. No predictable pattern for the placement of minicell-producing septa has yet been discerned in *E. coli* strains (ADLER et al., 1969; ADLER and HARDIGREE, 1972; TEATHER et al., 1974). Minicells can be produced at either pole of a cell and all possible sequences of forming central and minicell septa have been observed, e.g. cells may undergo several generations of division without the formation of a minicell, or several minicells may form sequentially at one pole in the absence of the formation of a central septum. To explain such observations, ADLER and HARDIGREE (1972) have proposed that cells possess three potential division sites, one in the central region of a cell, and one at either pole region. They propose that the sites at the polar region of a parent cell are destined to be located in the central region of progeny cells. Thus minicell formation would result from random premature activation of division sites located in the polar region. On the other hand, DONACHIE (1973) has suggested a different rationale for minicell formation. He proposes that sites for the location of central septa are usually shut off after division; however, if such sites remained active in the next generation, they would be located in the polar region and could subsequently give rise to a minicell-forming septum.

REEVE et al. (1973), MENDELSON and COYNE (1973), and COYNE and MENDELSON (1974) have studied the septum-forming characteristics of *B. subtilis* filamentous minicell-producing strains. In addition to the same combinations of sequences for forming minicell-producing and central septa as have been observed in *E. coli* strains, *B. subtilis divIV-A* and *divIV-B* strains can produce short, DNA-deficient rods that do not grow but do have the capacity for septum formation. REEVE et al. (1973) have observed that some of these rods divide one or two times, resulting in the production of two or three minicells. COYNE and MENDELSON (1974) have statistically analyzed the placement of septa in 46 clones of a *divIV-B* minicell-producing mutant observed by phase-contrast microscopy during the first 3 to 4 generations following spore germination. They observed that the probability of a minicell-producing septum being formed per septum-forming event is 0.31 and that a significant proportion (40%) of the minicells present in a clone occur in clusters of two or more minicells. Clusters of two minicells were often formed as a result of two minicell-producing divisions taking place one at each of two adjacent cell poles (which the authors designate as "sister poles" since they are produced by the same cell division event and therefore are the same age with respect to the generation during which they were formed). Furthermore, it was observed that clusters of minicells are most likely to be present at the two oldest cell poles in the clone (i.e. the most proximal and distal poles to the spore coat) and the two sister poles resulting from the first cell division. COYNE and MENDELSON (1974) suggest that the observed "preferential clustering" of minicells within a clone can be partially explained if the chance of a minicell-producing division increases as a cell pole becomes older. It is interesting to note that KHACHATOURIANS et al. (1973) have made similar observations of the clustering of minicell production at the oldest

poles of a clone during the burst of division that occurs when filaments of a *bug6*$^-$ minicell-producing strain are shifted from the non-permissive to permissive temperature. REEVE et al. (1973) and COYNE and MENDELSON (1974) have made the intriguing suggestion that the lack of chromosomal DNA in the polar region of *divIV-A* and *divIV-B B. subtilis* mutants may be related to the formation of minicell-producing septa. It will be interesting to see the relationship, if any, between minicell-production and the process of sporulation in *B. subtilis* strains.

It is apparent that the mechanism for selection of septum formation sites is yet to be elucidated in any minicell-producing system and it is unlikely that much progress will be made in this area until more information is available on the number and activities specified by different genes involved in the phenomenon of minicell formation (see Sect. II. C.). Evidence from a number of sources indicates that many minicell-producing strains have an increased resistance to ionizing radiation (see Sect. II. B.) and tend to be associated with filament formation (see Appds I. and III. A.) although it is not clear if these characteristics are truly pleiotropic effects of mutations that cause minicell production.

B. Radiation Sensitivity of *E. coli* and *S. typhimurium* Minicell-producing Strains

The *E. coli* minicell-producing mutant P678-54 of ADLER et al. (1966; 1967) was selected for its increased resistance to X-irradiation compared to its parent P678 while resistance to UV-irradiation remained the same for both strains. PATERSON and ROOZEN (1972b) have shown that an R plasmid-containing derivative of P678-54 (i.e. strain χ1009) is appreciably more resistant to γ-irradiation than is P678 and that this was due to the chromosomal mutations causing minicell production rather than the presence of the R plasmid. Minicell production in *E. coli* is due to two mutations at separate loci (*minA* and *minB*, ROOZEN and CURTISS, unpubl.; see Sect. II. C.), and FRALICK and HARDIGREE (unpubl.) have found that both mutations are necessary for increased X-ray resistance. HARDIGREE, KHACHATOURIANS, STALLIONS and ADLER (unpubl.) have examined the X-ray resistance of χ1260, a minicell-producing derivative of χ984 (a strain with a genetic background very different from P678-54; see Appdx II. B. 3. and Table 4), and of the TANKERSLEY (1970) *S. typhimurium* minicell-producer. Both strains show increased X-ray resistance compared to their respective non-minicell-producing parent strains. The minicell-producing strains of *S. typhimurium*, *S. anatum*, *S. enteritidis*, *S. pullorum*, *S. senftenberg*, and *S. worthington* isolated by EPPS and IDZIAK (1970) were selected by increased resistance to γ-irradiation. The resistance of these strains to X-ray and UV-irradiation has not been reported. These results strongly suggest that minicell-production and resistance to ionizing irradiation (i.e.X- and γ-irradiation) may be associated with the same mutations in *E. coli* and *Salmonella* species. Thus, it is apparent that ionizing-radiation resistance may be a promising

selection technique for minicell-producing mutants in other genera. It would be most interesting to examine the relationship between resistance to ionizing and UV-irradiation and minicell-production in the *B. subtilis*, *E. amylovora*, and *H. influenzae* strains.

C. Genetic Control of Minicell Production

Mutations at either of two different loci result in minicell formation in *B. subtilis* (REEVE et al., 1973). The *divIV-B1* mutant possesses a mutation closely linked to the *pheA* locus as determined by transduction with PBS1 bacteriophage and by co-transformation, while the mutation in the *divIV-A1* strain is cotransducible with the *ura* locus using the PBS1 phage. The minicells produced by both strains are similar, although the *divIV-B1* mutant produces more minicells than does the *divIV-A1* mutant. A third mutant (*divIV-B2*) was also isolated (REEVE et al., 1973) that possesses a mutation that may map at the same locus as the *divIV-B1* mutation since no wild-type recombinants were formed when these two strains were crossed.

The genetic control of minicell production by the *S. typhimurium* UT13 mutant has not yet been investigated. The strain forms recombinants with Hfr donor strains of *E. coli* K-12 and *S. typhimurium* LT-2 (SHEEHY, unpubl.) and is a prototroph lysogenic for at least three phages, one of which is immunologically related to phage P22 (SHEEHY et al., 1973a; CURTISS, unpubl.). There is no genetic information available on minicell production by the *Salmonella* strains isolated by EPPS and IDZIAK (1970). It would be most interesting to determine whether the same genetic locus is involved in minicell production in the *S. typhimurium* UT13 and ES878 strains of EPPS and IDZIAK (1970).

The *E. coli* minicell-producing strain P678-54 seems to have two mutant loci involved in the expression of the phenotype leading to minicell formation. ROOZEN and CURTISS (unpubl.) have conducted a diversity of conjugation experiments in an attempt to map these loci. The existence of five mutations affecting the Gal$^-$ phenotype in P678 (BACHMANN, 1972), some of which are suppressor mutations unlinked to the *gal* operon, has complicated linkage studies. The presence of amber suppressor mutations in χ925 (P678-54) has been confirmed by demonstrating that this strain supports the growth of various T4 amber mutants (FRAZER and CURTISS, unpubl.). Furthermore, aberrant recombinant yields were observed in crosses between χ925 and various *E. coli* donor strains, which seemed to indicate the presence of chromosome rearrangements in the P678 genetic background. Nevertheless, the ability of χ925 to produce minicells was lost when either the *lac-purE* (9 to 12 min) or *pyrC-trp* (24 to 27 min) region of the *E. coli* chromosome was introduced from wild-type donor strains. In matings with the F+ prototrophic minicell-producing strain χ964 (see Table 4 and Appdx II. B. 2.) and non-minicell-producing F$^-$ strains, the minicell-producing phenotype was only observed among recombinants that had inherited both the *lac*$^+$-*purE*$^+$ and *pyrC*$^+$ segments of the donor chromosome. It was also demonstrated that minicell-producing re-

combinants could be obtained by introducing the *lac*$^+$-*purE*$^+$ region from χ964 into P678, the parent to the *E. coli* minicell-producing mutant P678-54. Therefore, P678 already possessed one (*minB*) of the two mutations (*minA* and *minB*) necessary for minicell-production. It was subsequently shown that minicell-producing strains could be formed in two separate steps by using χ964 as the donor parent and selecting first Lac$^+$ recombinants which introduced the *minA* mutation and then PyrC$^+$ recombinants to introduce the *minB* mutation or vice versa (ROOZEN and CURTISS, unpubl.). Recently, KHACHATOURIANS (unpubl.) demonstrated that the *minA* locus is co-transducible with the *mtc* locus (i.e. at 10.5 min on the *E. coli* chromosome map of TAYLOR and TROTTER, (1972).

The ability of χ925 to produce minicells is also affected by other genetic changes that alter genes near the *minA* locus. FRALICK and CURTISS (unpubl.) found that many (but not all) T6^r mutants isolated from χ925 failed to produce minicells. It has also been found that the introduction of F *lacS* (MACRINA and BALBINDER, 1972) or F_{ts114}*lac* into χ925 eliminates the ability to produce minicells (MACRINA, unpubl.), although the introduction of other F′ *lac*$^+$ factors is without effect on minicell production (ROOZEN et al., 1971 a). In addition to these position effects, CURTISS (unpubl.) has found that the introduction of F′ ORF-1 (which carries the *lac*$^+$ *proC*$^+$ *T6*^{s} and *purE*$^+$ loci) into χ925 yields Lac$^+$ recombinants at a frequency of about 10^{-5} instead of the expected frequency of about 10^{-1}. The Lac$^+$ recombinant colonies were also very irregular in shape and none of them contained cells that were partially diploid for the *lac* to *purE* region. It was thus not possible to determine the dominance–recessive relationship of the alleles at the *minA* locus and it was suggested that such heterozygous partial diploids might result in cell lethality (CURTISS, unpubl.).

III. General Properties of Minicells and Studies of Minicells as a Population of Polar Regions of Cells

A. Introduction

Minicells constitute a unique *in vitro* system, one by definition incapable of division or growth, but possessing a functional cell wall, cell membrane, ribosomes, and an energy-generating system, and capable of maintaining the integrity of these systems for a considerable period of time. The experimental evidence on which these conclusions are based includes physiological, biochemical and electron-microscopic work with minicells from a number of species. Electron micrographs of thin sections through minicells of *E. amylovora* (HUANG and GOODMAN, 1970), marine pseudomonad B-16 (FORSBERG et al., 1970b), *E. coli* (ADLER and HARDIGREE, 1972; and Fig. 1) and *B. subtilis* (VAN ALSTYNE and SIMON, 1971; REEVE et al., 1973; and Fig. 2) show that the appearance of the cell envelope, plasma membrane, and ribosomes is like that of normal cells. The cell wall functions to protect minicells from lysis under a variety of conditions, as discussed more fully in Appendias III. B. and III. C., and Sect. III. C. TANKERSLEY (1970) and TANKERSLEY and WOODWARD (1973)

showed that there are no surface-antigenic differences between *S. typhimurium* minicells and normal cells by testing with absorbed antisera or antisera specific for O antigens. Furthermore, vaccines prepared with UV-irradiated or formalin-treated minicells had the same efficacy as vaccines prepared with formalin-killed normal cells, resulting in equivalent agglutination titers as well as excellent protection to mice subsequently challenged with live *S. typhimurium* cells. The intactness of the cell envelope is also indicated by the fact that bacteriophage can infect minicells of *E. coli* and *B. subtilis*, as discussed in Sect. V. Studies on active transport (Sect. III. G.), oxygen uptake and the generation of ATP (Sect. III. B.) indicate that the plasma membrane of minicells performs its varied functions as in normal cells. Evidence that minicell ribosomes are functional comes from studies of their capacity to synthesize protein in a cell-free system with artificial mRNA (FRALICK et al., 1969), and from the fact that plasmid-containing minicells and bacteriophage-infected minicells synthesize plasmid- and phage-specific proteins (see Sects. IV. G. and V.). Minicells are thus useful in studies of cell processes that continue in the absence of chromosomal DNA and of *de novo* synthesis of chromosome-encoded RNA and protein products. It is obvious that, since minicells are derived from the polar region of a cell, they should be useful as a means of characterizing the distribution of subcellular components between the ends and cylindrical regions of cells. In addition, with *E. coli* and *S. typhimurium* minicells, the effect of plasmids on the compartmentalization and activity of certain enzyme systems can be studied.

B. Energy Generation in Minicells

ADLER et al. (1967) demonstrated that *E. coli* minicells were active in oxygen consumption in minimal medium with glucose as a substrate and BLACK (1967) showed that *E. coli* minicells from cultures grown on glycerol were active in oxygen consumption with glycerol as a substrate. It has also been established that minicells of *B. subtilis* respire with glucose as a substrate and that respiration is coupled to phosphorylation with the generation of ATP as determined by the luciferin-luciferase assay (MENDELSON et al., 1974). The capacity of minicells to generate ATP is also evident from the observation by REEVE et al. (1973) that *B. subtilis* minicells are motile, and we have observed motility as well among *E. coli* and *S. typhimurium* UT13 minicells by phase-contrast microscopy. These studies suggest that minicells might be useful in studying chemotaxis and that motility does not depend on the presence of chromosomal DNA.

C. Maintainence of *E. coli* Minicells during prolonged Incubation at 4° C and 37° C or during Storage at —70° C or —190° C

The effect on a number of physiological functions due to the aging of purified *E. coli* minicell suspensions over a period of hours or days has been studied. BLACK (1967) measured the turbidity of minicell suspensions and their capacity

for respiration after incubation at 4° C in 3XD (FRASER and JERREL, 1953) growth medium or at 37° C in either 3XD or buffer. Penicillin was present in 3XD medium at 200 μg/ml to prevent growth of contaminating cells in minicell suspensions. When minicell suspensions were incubated in 3XD medium at 4° C, no loss of turbidity was observed during the 7 days of the experiment, while the turbidity of a cell suspension incubated under the same conditions decreased 4-fold. This indicates that, as with *B. subtilis* minicells (see Appdx III. B.), *E. coli* minicells are more resistant to autolysis than cells. When minicells were incubated for 2 days at 37° C in 3XD medium or buffer, the turbidity decreased gradually from an OD at 500 nm of 0.3 to 0.2, although the loss in turbidity was initially more rapid for minicells incubated in buffer. Hence autolysis of minicells was more immediate in buffer and was retarded when minicells were incubated in a growth medium. The capacity for respiration of minicells was determined by observing oxygen uptake in 3XD medium during a 2-h period. A minicell suspension incubated at 4° C in 3XD medium showed no decrease in capacity for respiration after 2 days and a decrease of only 10% after 7 days. However, minicells incubated at 37° C for 2 days showed a gradual decrease to 50% of the initial capacity for respiration and the rate of decrease was the same for suspensions in 3XD medium or buffer (BLACK, 1967). SILVERMAN (1967) observed that minicell suspensions incubated overnight at 4° C became noticeably more resistant to sonic disruption. To determine whether this observation was due to increased cell wall material, he measured the protein and polysaccharide content of minicell suspensions incubated for various periods of time at 4° C in 3XD medium. No change in total polysaccharide or protein content could be detected with the chemical methods employed. Similar observations have been made by FENWICK (unpubl.), who observed that minicells become more resistant to lysis by lysozyme treatment after refrigeration overnight and by LEVY (pers. comm.) who observed that minicells are more difficult to lyse after storage at 4° C or −20° C. The physiological basis of resistance to sonic disruption or lysozyme treatment as well as the possible reversibility of this phenomenon and its relationship, if any, to cold shock treatment remain undetermined. SILVERMAN (1967) also measured the loss in specific activity of NAD-dependent glycerol dehydrogenase and ribonuclease activities in minicell suspensions incubated at 4° C in 3XD medium. There was no decrease in the activity of glycerol dehydrogenase during the 24 h of the experiment and, since this enzyme is required for the utilization of glycerol as an energy source in respiration, these data are consistent with the observation of BLACK (1967) that no decrease in capacity for respiration is observed for at least 2 days under these conditions. Ribonuclease activity, however, decreased progressively at the same rate in minicell and cell suspensions with only 54 to 58% of the initial activity remaining after 8 h. KOOL et al. (1974) reported that after 2 days of storage at 2° C, minicells containing the Clo DF13 plasmid lost up to 80% of their capacity to synthesize RNA and protein. These studies indicate that different parameters of minicell suspensions (e.g. autolysis, respiration, various enzyme activities, and the capacity to synthesize RNA and

protein) decay at different rates, and that these decay or degradation processes are not necessarily related to one another.

KASS and YARMOLINSKY (1970) purified F'-containing minicells by sucrose-gradient centrifugation and then stored them at −70° C in tryptone broth containing 15% glycerol and 10^{-3} *M* $MgSO_4$. The capacity of the minicells to act as conjugal donors was preserved under these storage conditions. However, storing minicells overnight in the refrigerator resulted in a decrease in donor efficiency of more than 90%, which was partially reversible by incubation in broth (LEVY, 1971 b). INSELBURG and FUKE (1970) stored purified ColEl-containing minicells for 2 days at −70° C after quick freezing in a medium containing Tris, Casamino Acids, glucose and 7.5% glycerol. These minicells were competent to replicate ColEl after thawing.

KOOL et al. (1974) made a very interesting study of the capacity of minicells which contain the Col DF13 plasmid to incorporate precursors into DNA, RNA, and protein after storage at the temperature of liquid nitrogen (−190° C) for different periods of time. A linear decrease in the capacity for DNA synthesis was observed such that, after 4 to 6 days of storage, 50% of the initial synthesis capacity was lost. The capacity for RNA synthesis remained fairly constant for 4 days and then declined so rapidly that 50% of the initial capacity was lost by 8 days. The capacity for protein synthesis remained practically unchanged for at least as long as 16 days, hence, mRNA and the translation apparatus in these minicells remain stable under these storage conditions. It would be interesting to determine whether the proteins synthesized by minicells after this prolonged storage have the same molecular weight as when synthesized in fresh minicells.

D. Distribution of Stable Macromolecules in Minicells and Cells

A comparison of the distribution of stable macromolecules in cells and minicells of *B. subtilis*, *S. typhimurium* and *E. coli* is presented in Table 1. The amount of DNA is very low or below the level of detection in minicells of *B. subtilis* and *E. coli*, while in *S. typhimurium* minicells the amount of DNA is low compared to cells but higher than for minicells of *B. subtilis* and *E. coli*. This observation correlates with the observation by SHEEHY et al. (1973 a) that *S. typhimurium* UT13 minicells contain a plasmid of unknown function (called a cryptic plasmid). TUDOR et al. (1969) demonstrated by electron microscopy of thin sections that occasionally *E. coli* minicells of χ925 contain fractional portions of chromosomal DNA as a consequence of chromosomal material extending into the minicell-forming end of the cell at the time of septum formation. It was suggested that minicells containing a significant amount of chromosomal material might be found predominantly in the cell fraction after purification procedures based on differential centrifugation (TUDOR et al., 1969). The ratio mg RNA/mg protein in minicells of *B. subtilis* is very close to the value for cells, while that in *E. coli* minicells is 93 to 113% of the value for cells. SCANDLYN (1968) examined the stable RNA species in minicells and cells on

Table 1. Distribution of stable macromolecules in minicells and cells

Strain	Component	Minicells	Cells	Ratio MC/cell	Reference
B. subtilis divIV-A1	RNA	0.264 mg/mg protein	0.260 mg/mg protein	1.0	REEVE et al. (1973)
	DNA	not detectable	0.028 mg/mg protein	—	REEVE et al. (1973)
divIV-B1	RNA	0.230 mg/mg protein	0.234 mg/mg protein	0.99	REEVE et al. (1973)
	DNA	not detectable	0.029 mg/mg protein	—	REEVE et al. (1973)
S. typhimurium UT13	DNA	0.0034 mg/mg protein	0.037 mg/mg protein	0.09	TANKERSLEY (1970)
E. coli	RNA	0.36 mg/mg protein	0.32 mg/mg protein	1.1	ADLER et al. (1967)
		52 mg/g wet weight	56 mg/g wet weight	0.93	MICHAELS and TCHEN (1968)
		0.43 mg/mg protein	0.475 mg/mg protein	0.91	BLACK (1967); SILVERMAN (1967)
	DNA	<0.001 mg/mg protein	0.06 mg/mg protein	—	ADLER et al. (1967)
		0.15 mg/g wet weight	5.2 mg/g wet weight	0.029	MICHAELS and TCHEN (1968)
	putrescine	0.05 moles/ moles ribotide	0.06 moles/ moles ribotide	0.84	MICHAELS and TCHEN (1968)
	spermidine	0.017 moles/ moles ribotide	0.006 moles/ moles ribotide	2.8	MICHAELS and TCHEN (1968)

methylated albumin kieselguhr columns and determined that the ratio rRNA/tRNA was essentially the same in minicells (5.2) as in cells (5.6). MICHAELS and TCHEN (1968) examined the polyamine content of cells and minicells in order to determine whether the polyamines are preferentially associated with DNA. The total polyamine content was 0.14 moles *N*/mole RNA phosphate in cells and 0.15 in minicells, which showed that polyamines are not preferentially associated with DNA. When the polyamines were considered separately, it became apparent that the content of spermidine is enriched 2.8-fold in minicells. It would be interesting to know if the pathway for the synthesis of putrescine and spermidine is functional in minicells and to determine whether the enrichment for spermidine in minicells is due to subcellular compartmentalization or to differences in spermidine turnover in minicells compared to cells.

ALBERTS and FREY (unpubl. results cited in ALBERTS, 1970) examined by DNA-cellulose chromatography *E. coli* cells and DNA-deficient minicells for the relative levels of the more than 15 proteins that bind tightly to double-stranded *E. coli* DNA. Much lower amounts of these proteins were observed in minicell extracts, suggesting that these proteins, which include DNA and RNA polymerases, are associated with the chromosomal region of the cell *in vivo*.

E. Distribution of Enzymes in Minicells Compared to Cells

Most of the data available on enzyme activities in minicells have been obtained with the *E. coli* system and are presented in Table 2, which has been compiled from two sorts of studies: (1) determinations of the specific activities of various enzymes in cells and DNA-deficient minicells of *E. coli* by ADLER et al. (1967), SILVERMAN (1967), HURWITZ and GOLD, and SETLOW and COHEN (both cited in COHEN et al., 1968b), DVORAK et al. (1970), WICKNER et al. (1972), and ROGERSON and STONE (1974); (2) studies of the biological properties of plasmid-containing minicells from which the presence or absence of a number of enzyme activities in minicells may be inferred (also see Sect. IV.). Enzymes are grouped in Table 2 either by their location in one of two subcellular compartments (i.e. the periplasmic space or cytoplasm) or by their association with RNA or DNA metabolism. The specific activities of various enzymes in minicells and cells are not given in Table 2, instead, the ratios of the specific activity in minicells to the specific activity in cells have been computed and included in the table. Hence, a number greater than unity is obtained if the enzyme level (i.e. specific activity) is higher in minicells than in cells, while a value less than unity is obtained if the enzyme activity level is lower in minicells than in cells.

The periplasmic space is defined as the region between the outer membrane of the cell envelope and the inner plasma membrane and enzymes located in this space are released from cells by osmotic shock or by forming spheroplasts. DVORAK et al. (1970) attempted to use minicells to determine whether enzymes located in the periplasmic space are concentrated at the polar region of the cell in so-called polar caps. The results presented in Table 2 indicate that all enzymes in the periplasmic space have a higher activity in minicells relative to cells for minicells obtained from log- or stationary-phase cultures, except ribonuclease I which has a higher relative activity in minicells from log-phase cultures and a lower activity in minicells from stationary-phase cultures. For the cytoplasmic enzymes assayed in this study (i.e. β-galactosidase, glutamine synthetase, inorganic pyrophosphatase, polynucleotide phosphorylase and ribonuclease II) the specific activities in minicells were 0.7 to 1 times the specific activities in cells. It should also be noted that the relative specific activities of β-galactosidase obtained by two different laboratories show good agreement (ADLER et al., 1967; DVORAK et al., 1970). It is interesting that although 5'-nucleotidase was enriched in the periplasmic space of minicells, the cytoplasmic inhibitor of 5'-nucleotidase was very low in minicells. DVORAK et al. (1970) concluded that periplasmic enzymes are generally enriched at the poles of cells. However, this conclusion is open to some reinterpretation because enzymes in the periplasmic space should have a higher specific activity in minicells since the surface-to-volume ratio is higher for minicells than for cells, as can be calculated from the average dimensions of *E. coli* minicells (0.70 μm diameter) and cells (2.8 $\times$ 0.83 μm) determined by electron microscopy (FRAZER and CURTISS, 1973). Hence, the surface-to-volume ratio for minicells is 8.6 μm^{-1} and that for cells is 5.5 μm^{-1}, which demonstrates that the surface-to-volume ratio for minicells is 1.5 times that of cells. Therefore, the ratio of

Table 2. Distribution of some enzymes in E. coli minicells[a]

Enzyme	Enzyme detection	Data[b]	Kind of minicells	Reference
Enzymes of the periplasmic space:				
5′-nucleotidase	assay	2.3 (log)	925 Pla⁻	Dvorak et al. (1970)
acid hexose phosphatase	assay	3.5 (log)	925 Pla⁻	Dvorak et al. (1970)
cyclic phosphodiesterase	assay	1.5 (log), 2.5 (stationary)	925 Pla⁻	Dvorak et al. (1970)
ribonuclease I	assay	1.5 (log), 0.7 (stationary)	925 Pla⁻	Dvorak et al. (1970)
alkaline phosphatase	assay	1.6 (stationary)	925 Pla⁻	Dvorak et al. (1970)
	assay	7.3 (early stationary)	925 Pla⁻	Silverman (1967)
Cytoplasmic enzymes:				
NAD-dependent glycerol dehydrogenase	assay	0.40 (early stationary)	925 Pla⁻	Silverman (1967)
inosine monophosphate dehydrogenase	assay	0.10 (early stationary)	925 Pla⁻	Silverman (1967)
β-galactosidase	assay	0.7 (log and stationary)	925 Pla⁻	Dvorak et al. (1970)
	assay	0.77	925 Pla⁻	Adler et al. (1967)
glutamine synthetase	assay	0.7 (log and stationary)	925 Pla⁻	Dvorak et al. (1970)
inorganic pyrophosphatase	assay	1.0 (log and stationary)	925 Pla⁻	Dvorak et al. (1970)
inhibitor of 5′-nucleotidase	assay	0.009 (log and stationary)	925 Pla⁻	Dvorak et al. (1970)
polynucleotide phosphorylase	assay	activity in cells and MCs equivalent	925 Pla⁻	Dvorak et al. (1970)
RNA-related enzymes:				
ribonuclease II	assay	activity in cells and MCs equivalent	925 Pla⁻	Dvorak et al. (1970)
ribonuclease (total)	assay	1.2	925 Pla⁻	Silverman (1967)

Table 2 (continued)

Enzyme	Enzyme detection	Data[b]	Kind of minicells	Reference
DNA-dependent RNA polymerase	assay	0.012	925 Pla⁻	Hurwitz and Gold cited in Cohen et al. (1968b)
	inference	present in Pla⁺ MCs which are proficient in:		
		uracil and AA incorporation	984 (R222)	Levy (1971a)
		AA incorporation	925 (R6-3*drd*12)	Cohen et al. (1971b)
		AA incorporation	925 (Clo DF13)	Kool et al. (1972)
		Urd and AA incorporation	925 (ColV), 925 (Col-*trp*), 925 (R64-11)	Roozen et al. (1971b)
		Urd and AA incorporation	925 (219)	van Embden and Cohen (1973)
		Urd and AA incorporation	925 (Clo DF13)	Kool et al. (1974)
		Synthesis of *trp* mRNA	925 (Col-*trp*)	Roozen et al. (1971b)
		de novo anthranilate synthase synthesis	925 (Col-*trp*)	Frazer and Curtiss (1973)
β and β' subunits of RNA polymerase	SDS PAGE	present in equivalent amounts in Pla⁻ and Pla⁺ MCs	925 Pla⁻, 925 (R64-11)	Rogerson and Stone (1974)
RNA methylase	assay	0.77	925 Pla⁻	Hurwitz and Gold cited in Cohen et al. (1968b)
DNA-related enzymes:				
DNA methylase	assay	<0.04	925 Pla⁻	Hurwitz and Gold cited in Cohen et al. (1968b)
DNA ligase	assay	0.77	925 Pla⁻	Hurwitz and Gold cited in Cohen et al. (1968b)
	inference	present since MCs repair γ- and X-ray-induced single-strand breaks	925 (R64-11), 925 (λdv)	Roozen et al. (1971d), Paterson and Roozen (1972b), Paterson et al. (1973)
	inference	present since replication occurs in MCs containing:		

		ColE1	925 (ColE1)	INSELBURG (1970), INSELBURG and FUKE (1971), INSELBURG (1973)
		R222	925 (R222)	INSELBURG (1971)
		ColV	925 (ColV)	ROOZEN et al. (1971a)
DNA polymerase I	assay	0.45	925 Pla⁻	HURWITZ and GOLD cited in COHEN et al. (1968b)
	inference	present since replication of ColE1 occurs in MCs	925 (ColE1)	References as for DNA ligase
	inference	present since MCs show fast repair of γ-irradiation-induced and single-strand breaks	925 (R64-11), 925 (λdv)	PATERSON and ROOZEN (1972b), PATERSON et al. (1973)
DNA polymerase II	assay	0.5 (approximately)	925 Pla⁻	WICKNER et al. (1972)
UV endonuclease	inference	low activity detectable by nicking of CCC DNA after UV-irradiation	925 (R64-11)	PATERSON and ROOZEN (1972b)
photoreactivating enzyme	assay	not detectable	925 Pla⁻	SETLOW and COHEN cited in COHEN et al. (1968b)
	inference	MCs have half the activity of cells by rate of light repair after UV-irradiation	925 (R64-11)	PATERSON and ROOZEN (1972b)
exonuclease V	assay	activity in cells and MCs apparently equivalent	925 Pla⁻, 925 (λdv), 925 (R64-11)	PATERSON and VAN DORP (in preparation, 1974)
deoxyribonuclease (ATP-independent)	assay	0.125 (early stationary)	925 Pla⁻	SILVERMAN (1967)
deoxyribonuclease	inference	present since MCs degrade conjugally transferred DNA	925 Pla⁻, 925 (R100), 925 (R100-1), 925 (F'KLF-1)	SHEEHY (1972), SHEEHY et al. (1972b), KHACHATOURIANS et al. (1972, 1974)
deoxyribonuclease triggered by colicin E2 action	inference	present since kinetics of loss of CCC DNA in MCs and cells are the same after colicin E2 treatment	925 (R64-11)	KHACHATOURIANS and RIDDLE (1973)

[a] Abbreviations used in this table are: Pla⁻, non-plasmid-containing; Pla⁺, plasmid-containing; MCs, minicells; AA, amino acid; Urd, uridine; SDS PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; CCC, supercoiled covalently closed circular.

[b] Numbers presented in this column are the ratio of the specific activity for minicells to the specific activity for cells and are calculated from the specific activities reported in the designated reference. If it has been reported whether minicells were from cultures grown to log or stationary phase, this information is included in parentheses.

specific activity of enzymes for minicells compared to cells should be greater than 1.5 to demonstrate an increased level of periplasmic enzymes at the polar region. It is evident from the data in Table 2 that for periplasmic enzymes a ratio of minicell to cell activity greater than 1.5 was observed by DVORAK et al. (1970) only for 5'-nucleotidase and acid hexose phosphatase in minicells from log-phase cultures and for cyclic phosphodiesterase in minicells from stationary-phase cultures. The variations in relative enzyme levels depending on the culture age are interesting and suggest that minicells might be useful in examining the compartmentalization of other surface components and of enzymes involved in cell wall synthesis as well as in examining variations in the distribution of components in these surface compartments with culture age.

SILVERMAN (1967) also did an interesting study on the levels of enzymes in minicells and cells. The four enzymes assayed were chosen because different subcellular distributions could be postulated for most of them: alkaline phosphatase is in the periplasmic space, glycerol dehydrogenase and ribonuclease activities might be distributed evenly throughout cells, and deoxyribonuclease and inosine monophosphate dehydrogenase might be associated with the chromosomal region of the cell and thus be very low in the polar region of cells. The periplasmically located enzyme, alkaline phosphatase, was quite high in minicells relative to cells (7.3) in these experiments and indicated, in contrast to the data of DVORAK et al. (1970), a preferential location at the cell poles. Glycerol dehydrogenase activity was significantly lower in minicells than in cells and, since aging studies showed that this enzyme was stable in minicells for at least 24 h at 4° C (Sect. III. C.), it is probable that the lower level in minicells reflects the intracellular distribution of the enzyme, i.e. that less glycerol dehydrogenase is distributed to the polar regions of cells than to the cylindrical regions. Total ribonuclease activity for minicells was equivalent to that in cells, but the activities of different ribonucleases were not determined. Total deoxyribonuclease activity and inosine monophosphate dehydrogenase, which is in the *de novo* pathway for GMP synthesis, were considerably lower in minicells than in cells. This indicates that in addition to some enzymes which have DNA as their substrate (HURWITZ and GOLD cited in COHEN et al., 1968b), certain nucleotide biosynthetic enzymes may tend to be located close to DNA within cells. This idea is supported by some aspects of the studies by KHACHATOURIANS and CURTISS (unpubl. results) on the conversion of thymine and thymidine to phosphorylated nucleotides by DNA-deficient and plasmid-containing minicells. TMP appeared within 15 sec after the addition of exogenous thymidine to DNA-deficient or plasmid-containing minicells, and TDP and TTP appeared between 15 and 30 sec. The conversion of exogenous thymine into nucleotides was low even in plasmid-containing minicells although, interestingly enough, the amount of thymidine formed was about 4 times higher in plasmid-containing minicells than in DNA-deficient minicells. It is significant that most exogenous thymidine was degraded to thymine and deoxyribose-1-phosphate by thymidine phosphorylase in minicell suspensions and that inhibitors of this reaction (e.g. deoxyadenosine) were effective in suppressing this

conversion. It would be of interest to know the metabolic fate of exogenous thymine or thymidine in minicells from *thy*⁻ strains. Studies on the conversion of uracil and uridine to nucleotides have not been reported.

A great deal of interest has been focused on the DNA-dependent RNA polymerase activity of minicells. HURWITZ and GOLD (cited in COHEN et al., 1968b) showed that the level of this enzyme is extremely low in DNA-deficient minicells and the deficiency of RNA polymerase in these minicells was further indicated by the observation of FRALICK et al. (1969) and FRALICK (1970) that DNA conjugally transferred into minicell recipients was not transcribed into RNA nor was protein synthesized. FRALICK et al. (1969) showed that extracts from DNA-deficient minicells were capable of supporting *in vitro* protein synthesis when provided with an artifical poly(U) message and thus that the deficiency of minicells in RNA synthesis after conjugation was due primarily to the absence of RNA polymerase. The exciting observations that plasmid-containing minicells are proficient in RNA and protein synthesis (see Sect. IV. G.) indicated that active RNA polymerase molecules might be closely associated with plasmid DNA and segregate with plasmid DNA into minicells. LEVY (1971a) examined the question of whether the RNA polymerase activity associated with plasmid-containing minicells was of host-cell origin or encoded by the plasmid genome in a manner analogous to some bacteriophages. For example, T3 and T7 phages specify the synthesis of a new RNA polymerase resistant to rifampin (a semisynthetic derivative of rifamycin SV, also known as rifampicin) unlike the drug-sensitive wild-type *E. coli* RNA polymerase of host cells. LEVY (1971a) used the plasmid R222 (i.e. R100) and examined the rifampin sensitivity of plasmid-specific transcription in minicells purified from minicell-producing strains that were sensitive, resistant, or partially resistant to 100 μg/ml of rifampin. No differences between cells and minicells in sensitivity to the drug were apparent with rifampin-sensitive or -resistant strains. However, in strains partially inhibited by 100 μg/ml, it was observed that minicells were more sensitive to rifampin (i.e. incorporation was inhibited by 80%) than were cells (i.e. inhibited by 50%). The interpretation of all these results was that plasmid transcription is carried out by host RNA polymerase although there may be some subtle differences between the plasmid and the *E. coli* chromosome with respect to the interaction of RNA polymerase and DNA. ROGERSON and STONE (1974) have examined DNA-deficient and plasmid-containing minicells for the presence of β and β' subunits of RNA polymerase by SDS polyacrylamide-gel electrophoresis of whole-cell extracts and have shown that these two types of minicells contain similar amounts of these subunits. The authors feel that this observation supports the concept indicated by the work of MATZURA et al. (1973) and DALBOW (1973) that some sort of inactive RNA polymerase is present free of DNA in cells and consequently is found in DNA-deficient minicells. CORNETT and REEVE (1974) have shown that *B. subtilis divIV-B* DNA-deficient minicells infected with the phage SPO1 synthesize *de novo* RNA and protein (see Sect. V.). These observations indicate that these *B. subtilis* minicells, in apparent contrast to *E. coli* minicells, may contain active

RNA polymerase. Alternatively, the SPO1 phage may inject either a sigma factor to activate existing β and β' subunits or an intact functional RNA polymerase.

In addition to RNA polymerase, HURWITZ and GOLD (cited in COHEN et al., 1968b) also assayed RNA methylase, DNA methylase, DNA ligase and DNA polymerase I activity in DNA-deficient minicells. DNA methylase activity was very low, if present at all in minicells, indicating that this enzyme may be closely associated with DNA and thus have a distribution apparently similar to that of functional RNA polymerase. DNA methylase activity has not been determined in plasmid-containing minicells. RNA methylase and DNA ligase appeared to be distributed randomly throughout the cell like β-galactosidase, while DNA polymerase I appeared to have a distribution like that observed for glycerol dehydrogenase by SILVERMAN (1967), indicating that DNA polymerase I is not restricted to the chromosomal region of a cell. Other indications that DNA ligase and DNA polymerase I are present and functioning in minicells comes from various studies with plasmid-containing minicells which are treated in detail later in this review (Sect. IV.). WICKNER et al. (1972) have demonstrated that DNA-deficient minicells also contain DNA polymerase II, which is present at half the specific activity observed in cells. As in cells, the level of DNA polymerase II in minicells is about 10% of the total activity due to DNA polymerase I and II. Thus, the great similarity between DNA polymerases I and II in their subcellular distribution suggests a role in repair processes for DNA polymerase II by analogy with DNA polymerase I (WICKNER et al., 1972). Photoreactivating enzyme was not detectable in DNA-deficient minicells by direct assay (SETLOW and COHEN cited in COHEN et al., 1968b); however, plasmid-containing minicells are capable of light repair of UV-irradiation damage at half the rate observed in cells (PATERSON and ROOZEN, 1972b). Hence the presence of this enzyme, like active RNA polymerase, may depend on segregation to minicells in association with plasmid DNA. Studies on plasmid-containing minicells and their repair capacity after ionizing and UV-irradiation indicate that minicells can repair many kinds of damage (see Sect. IV. E.); however, UV-endonuclease activity is apparently very low even in plasmid-containing minicells.

A variety of deoxyribonuclease activities seem to be present in DNA-deficient and plasmid-containing minicells. SILVERMAN (1967) demonstrated by direct enzyme assay that deoxyribonuclease activity was present in DNA-deficient minicells although at about 10% of the level found in cells. The presence of deoxyribonuclease activity in DNA-deficient minicells has also been demonstrated in studies on the degradation of DNA transferred to minicell recipients after conjugation (SHEEHY et al., 1972b; SHEEHY, 1972; KHACHATOURIANS et al., 1972, 1974) as described more fully in Sect. VI. B. 1. PATERSON and VAN DORP (1974) have done a comparative study of the ATP-dependent exonuclease V activity (encoded by the *recB* and *recC* genes) in DNA-deficient minicells and minicells containing either the plasmid R64-11 or λdv (this latter strain was also *recA*$^-$). Exonuclease V activity in DNA-deficient minicells was

approximately the same as observed in cells while the activity in plasmid-containing minicells was significantly lower. It was suggested that plasmid DNA might compete with the ^{32}P-labeled DNA used in the enzyme assay and thus spuriously lower the observed activity. Some interesting work has been done on deoxyribonuclease activities in plasmid-containing minicells following treatment with colicin E2 (KHACHATOURIANS and RIDDLE, 1973, and discussed in Sect. IV. F.). With the exception of exonuclease V, specific deoxyribonuclease activities have not been assayed in DNA-deficient or plasmid-containing minicells and there is no information yet on whether some of these enzymes segregate to minicells with plasmid DNA.

F. Membrane and Cell Wall Biosynthesis

Minicells as a preparation of cell ends have been used to compare the distribution of membrane markers in polar regions of the cell as opposed to the distribution in whole cells. Two complementary studies (WILSON and FOX, 1971; GREEN and SCHAECHTER, 1971, 1972) utilizing this approach support a dispersive model of membrane synthesis for *E. coli* in which membrane units appear to be synthesized throughout the membrane.

GREEN and SCHAECHTER (1972) using χ984 examined the dilution of membrane markers with time. A culture was prelabeled for more than a generation with a marker for either membrane phospholipid ([2-^{3}H]glycerol) or a membrane protein (the heme precursor, [^{3}H]δ-aminolevalinic acid) in the presence of a steady-state protein label ([^{14}C]histidine) that was subsequently maintained throughout the experiment. Minicells and cells were separated and most of the cells reincubated for further growth in the absence of membrane label. Growth was continued for up to 5 generations with 3 to 5 additional cycles of separating newly formed minicells and reincubating the cells. The decrease in the ratio of ^{3}H/^{14}C with time was exponential and the rate of dilution of ^{3}H counts was the same for minicells and cells regardless of whether membrane phospholipid or membrane protein had been labeled. At any given time, the ratio of ^{3}H/^{14}C was higher for minicells than for cells. This is expected since minicells have a surface-to-volume ratio approximately 1.5 times higher than cells. When the experiment was repeated with a pulse of [2-^{3}H]glycerol and the minicells separated initially after one fourth of a generation, a constant decrease in the ^{3}H/^{14}C ratio was still observed (GREEN and SCHAECHTER, 1971, 1972).

WILSON and FOX (1971) prepared a derivative of χ925 that is *lac*$^+$ and *bgl*$^+$ (i.e. has the capacity for β-galactoside and β-glucoside utilization) for their studies and did two kinds of experiments. First they observed the distribution of whole-membrane protein when cultures were prelabeled with [^{3}H]isoleucine before separating minicells and shifting the cells to medium with [^{14}C]isoleucine for further growth. At various times minicells were isolated and most of the cells reincubated. Membrane fractions were prepared by sonication and differential centrifugation. The ^{3}H cpm/μg membrane protein for either ^{3}H or ^{14}C were identical for minicell and cell membranes at a given time. Thus, the

distribution of total membrane proteins is the same in cells and minicells. The second experiment examined the distribution of two specific permeases. The culture was preinduced only for β-glucoside transport, minicells and cells were separated and the cells shifted to a medium to induce exclusively β-galactoside transport. As before, minicells were separated at various times and most of the cells reincubated. The ratio of transport activity to that of the corresponding hydrolase (phospho-β-glucosidase or β-galactosidase) was observed for minicells and cells. The ratios for the β-glucoside and β-galactoside transport systems were different from each other but constant in minicells and cells throughout the experiment. This supports the concept that permeases are distributed as randomly in the membrane as soluble cell protein is in the cell. The ratios of transport to enzyme activity were the same for minicells and cells, in contrast to the higher ratio of membrane components to total protein observed for minicells by GREEN and SCHAECHTER (1972). This discrepancy does not invalidate the conclusion drawn by WILSON and FOX (1971), rather the results indicate the inapplicability of their assumption that the distribution of a specific enzyme in minicells is the same as the distribution of soluble cell protein. In particular, it has been demonstrated that the activity of β-galactosidase can be lower in minicells than in cells (ADLER et al., 1967; DVORAK et al., 1970; Table 2, p. 17).

It is evident that minicells could be profitably used to examine the distribution of many other specific plasma-membrane and cell-envelope components that may partition differently between the ends and cylindrical regions of rod-shaped cells. Indeed TEATHER and GOODELL have evidence that some proteins of the outer membrane of the cell envelope in *E. coli* are different at the cell poles and the cylindrical regions of cells (cited in DONACHIE, 1973; no statement was made as to whether minicells were utilized in this study). REEVE and MENDELSON (1973b) described the adsorption of the phages SP01, SP17 and ϕ29 to minicells of *B. subtilis* (see Sect. V.) and suggested that minicell systems could be used to screen for phages with adsorption sites located exclusively in polar regions or cylindrical regions of cells.

In addition, minicells and minicell-producing strains promise to be quite useful in testing various hypotheses of cell wall synthesis and organization. SCHWARZ and ASMUS (1969) used low levels of penicillin (10–50 units/ml) to obtain specific interruption of cell septum formation in *E. coli* such that instead of septa, bulges were produced. With a minicell-producing strain, in contrast to other strains, cell bulges were often displaced to cell poles. *E. coli* minicell-producing strains that require the specific cell wall component, diaminopimelic acid, can be prepared (FRAZER, unpubl.; see Appdx II. B. 4., and Table 4) and should be useful in studies of cell wall biosynthesis. MENDELSON and REEVE (1973) have studied growth zones for cell elongation in *B. subtilis* strains that are *tag-1* (i.e. form a thickened, irregularly shaped, teichoic acid-deficient cell wall resulting in Rod⁻ morphology at the nonpermissive temperature) and also either *divIV-A*⁻ or *divIV-B*⁻, and which therefore produce filamentous minicell-forming cells. Careful growth studies have shown that at the nonpermissive

temperature the Rod$^-$ morphology is produced only in regions of the cell wall undergoing elongation and that regions of Rod$^+$ morphology are conserved. Septation occurs in both Rod$^-$ and Rod$^+$ cell wall regions so that minicell production continues at the nonpermissive temperature. The earliest site of cell elongation is subterminal and any possible relationship between this site and minicell formation remains obscure.

G. Membrane Transport Studies Using Minicells of *E. coli* and *B. subtilis*

VALLÉE et al. (1972) demonstrated that *E. coli* DNA-deficient minicells transport [^{3}H]leucine into minicells, presumably by active transport, although this point has not been tested. This membrane function, as well as many other cell functions, is blocked by the action of T4 bacteriophage ghosts on cells. Treating minicell suspensions with T4 ghosts at a multiplicity of infection of 3 for three minutes before adding [^{3}H]leucine resulted in almost complete inhibition of leucine transport, showing that at least one effect of T4 ghosts on cells occurs in the absence of chromosomal DNA.

REEVE and MENDELSON (1974) have studied the capacity of *B. subtilis* minicells to transport labeled uracil, thymine and 12 different amino acids. The uptake of amino acids in *B. subtilis* cell suspensions was linear and most of the label found in cells was acid-precipitable. In contrast, the transport observed with minicells was biphasic with an initial rapid rate followed by a very slow rate of accumulation. Furthermore, essentially no acid-precipitable label was present in minicells. The soluble amino acid pool sizes were different for minicells and cells. The pool size was larger in minicells for methionine, histidine, proline, valine and glutamic acid, but smaller in minicells for aspartic acid, alanine, phenylalanine, isoleucine, arginine, glycine and serine. The rate of amino acid accumulation was higher for cells than for minicells and in some instances the difference in rates was quite marked; for example, cells had a rate of arginine uptake that was about 14 times higher than minicells. These quantitative differences between the transport capabilities of minicells and cells may reflect a diversity in the subcellular location of various transport systems as between polar and cylindrical regions and/or differences in turnover among the various transport systems. More detailed studies on the transport and maintenance of a pool of proline in minicells indicated that energy metabolism is required for both processes, since azide or iodoacetate inhibited transport and resulted in the slow loss of proline from preloaded minicells. It was also established that minicells concentrated proline so that the internal proline concentration was 150 times higher than the external concentration and that internally accumulated proline was accessible to an energy-dependent exchange with exogenous proline or glutamic acid, but not with exogenous arginine. Minicells also were shown to transport uracil in a manner similar to the amino acids whereas thymine was not transported. In a separate study, REEVE and MENDELSON (1973a) demonstrated that treating *B. subtilis* cells or minicells with pronase at a concentration that has no effect on cell growth (250 μg/ml)

significantly decreased the rate of uptake of labeled proline or isoleucine and also resulted in the release of amino acid from prelabeled cells or minicells. The kinetics of release of accumulated amino acid was different for minicells and cells. Maximum release from minicells occurred within 10 min, after which the new plateau remained constant; the amount of label released was different for the two amino acids, i.e. 35% of the labeled proline was released *vs.* 65% of the labeled isoleucine. By contrast, the loss of label from cells was continuous and gradual, although amino acid continued to be incorporated into acid-precipitable material, indicating that soluble amino acid pool sizes were decreased in cells as well as in minicells. REEVE and MENDELSON (1973a) suggest that these results are consistent with the hypothesis that pronase treatment releases amino acid-binding sites from the surface of *B. subtilis* cells and minicells and therefore is potentially very useful for studying binding proteins of gram-positive organisms. Recently MENDELSON et al. (1974) reported that minicells of *B. subtilis* form protoplasts after treatment with lysozyme and that these minicell protoplasts concentrate proline against a concentration gradient.

IV. Studies of Plasmid-containing Minicells

A. Discovery and Proof that DNA Present in Minicells of Plasmid-bearing Strains is Plasmid DNA

The discovery that plasmid-containing minicell-producing strains form minicells containing the same plasmid as parent cells was reported by several laboratories in 1970 (INSELBURG, 1970; KASS and YARMOLINSKY, 1970; ROOZEN et al., 1970; LEVY, 1970; LEVY and NORMAN, 1970). These discoveries were based on the preliminary observations that minicells from plasmid-containing strains grown for numerous generations in the presence of radioactive thymidine or thymine contained much more acid-insoluble label than did minicells from nonplasmid-containing strains (INSELBURG, 1970; KASS and YARMOLINSKY, 1970; LEVY and NORMAN, 1970; ROOZEN et al., 1970, 1971a, c; COHEN et al., 1971a, b; KOOL et al., 1972). The labeled material in these minicells could be confirmed as DNA by its susceptibility to deoxyribonuclease (KASS and YARMOLINSKY, 1970; ROOZEN et al., 1971a; LEVY, 1971a). Many different physical, biochemical and biological techniques have been used to characterize the DNA in these minicells and to prove that it is plasmid DNA.

1. Enrichment for Supercoiled DNA in Minicells

Physical characterization of the DNA present in minicells from plasmid-bearing strains generally indicates that a significant percentage is composed of supercoiled, covalently closed, circular (CCC) DNA, a form of DNA known to be associated with plasmids (see CLOWES, 1972). Therefore, the demonstration that CCC DNA is enriched in minicells compared to cells and is absent from

DNA-deficient minicells is good evidence that the minicell DNA is plasmid DNA. The presence of CCC and other forms of DNA may be demonstrated on three types of gradients that are summarized below.

a) CsCl–ethidium bromide buoyant density-gradient centrifugation distinguishes among three types of DNA structures by means of the differential binding of ethidium bromide (HUDSON and VINOGRAD, 1967): CCC DNA bands at the highest density, catenated DNA composed of interlocking CCC and open circular (OC) DNA molecules bands at intermediate density, and double-stranded linear and OC DNA band at the lowest density.

b) Neutral sucrose velocity sedimentation distinguishes among DNA species by differences in size and shape, and four classes of DNA species can be demonstrated (HUDSON and VINOGRAD, 1969): catenated molecules sediment most rapidly, then CCC monomers, then OC monomers, and finally linear DNA.

c) Alkaline sucrose velocity sedimentation distinguishes between CCC DNA, which forms a compact structure in alkali and therefore sediments rapidly, and denatured single-stranded DNA, which may be derived from OC DNA or sheared linear DNA (FREIFELDER, 1968).

The percentage of CCC DNA recovered from purified minicells of plasmid-bearing strains labeled during growth is at least 20 times higher than that obtained from cells, except for minicells containing F and F′ plasmids in which very little CCC DNA has been demonstrated (KASS and YARMOLINSKY, 1970; ROOZEN, 1971). The small amount of DNA derived from a purified minicell preparation of the DNA-deficient strain χ925 consists of linear molecules with no CCC DNA present (COHEN et al., 1971 b; VAN EMBDEN and COHEN, 1973). CCC DNA has been demonstrated in minicells produced by cells containing: (a) repressed and derepressed conjugative class-1 R plasmids (i.e. two replicons contain transfer and resistance determinants that are covalently linked to form a cointegrate) (CLOWES, 1972; LEVY and NORMAN, 1970; ROOZEN et al., 1971 a; COHEN et al., 1971 b; LEVY, 1971 a, b); (b) a class-2 R factor (i.e. replicons contain transfer and resistance determinants that are not covalently linked) consisting of the transfer plasmid I and the tetracycline-resistance plasmid 219 or bearing just the 219 plasmid (VAN EMBDEN and COHEN, 1973); (c) both nonconjugative and repressed and derepressed conjugative bacteriocinogenic plasmids (INSELBURG, 1970; ROOZEN et al., 1971 a; KOOL et al., 1972); (d) the *λdv* plasmid (ROOZEN et al., 1971 c; ROOZEN, 1971). The percentage of DNA found in the supercoiled form varies somewhat with different plasmids (ROOZEN et al., 1971 a) as well as with the lysis and centrifugation techniques employed. For example, INSELBURG (1970) found 15% of ColE1 in the CCC form by CsCl–ethidium bromide centrifugation but 80% by neutral sucrose sedimentation. The low percentage of CCC DNA observed with F- and F′-containing minicells remains unexplained (see Sect. VI. C. of this review for further discussion). The demonstration that minicells contain CCC DNA does not establish the identity of that DNA with the plasmid contained in parent cells, and additional techniques have been used to prove this identity.

2. Sedimentation Coefficients and Contour Lengths of DNA from Minicells

INSELBURG (1970) demonstrated that the DNA extracted from purified minicells obtained from a continuously labeled culture had an *s* value in neutral sucrose gradients that was very similar to the *s* value reported for ColE1 supercoiled DNA (24S). It was subsequently shown that the contour length of ColE1 DNA from minicells was 2.31 ± 0.06 μm (INSELBURG and FUKE, 1970) which agreed with the previously determined contour length of 2.33 ± 0.06 μm for ColE1 DNA from cells. INSELBURG (1971) has also demonstrated that the *s* value and contour length of DNA from R222 (i.e. R100) plasmid-containing minicells are the same as those found for R222 DNA obtained from cells. COHEN et al. (1971a) analyzed the peaks obtained with CsCl–ethidium bromide centrifugation of DNA extracted from minicells for their sedimentation properties in neutral sucrose and found *s* values consistent with those expected for the CCC and OC DNA forms of the R6(3) plasmid contained in parent cells. VAN EMBDEN and COHEN (1973) demonstrated that the DNA extracted from purified minicells of a strain containing the class-2 R plasmid aggregate (I + 219) had the same sedimentation pattern as DNA from another strain (W3110N) carrying this class-2 R plasmid aggregate, i.e. there were three peaks evident with approximate values of 60S, 42S, and 27S. DNA extracted from minicells of a strain containing only the 219 plasmid exhibited just one peak at about 27S on neutral sucrose gradients. KOOL et al. (1972) demonstrated that the CCC DNA obtained from minicells of a Clo DF13-containing strain on CsCl–ethidium bromide gradients was composed of two sizes of DNA, i.e. 24S and 18.6S, by neutral sucrose sedimentation. It was further demonstrated that the 18.6S species is derived from the 24S species by treatment with deoxyribonuclease I (pancreatic) and that the contour length of Clo DF13 DNA by electron microscopy in minicells and cells is 3.1 ± 0.2 μm.

The *S. typhimurium* minicell producer has two cryptic plasmids, a large plasmid (130×10^6 daltons) and a small plasmid (2.5×10^6 daltons). Only the small plasmid is found in minicells as observed by electron microscopy of the CCC DNA fraction of purified minicells isolated from CsCl–ethidium bromide gradients (SHEEHY et al., 1973a). In spite of the fact that R⁻ *Salmonella* minicells contain cryptic plasmid DNA, some significant information about R plasmid biology has been obtained using these minicells. Both R100-1 and R64-11 DNA segregate to these minicells. Furthermore, it has been determined by neutral sucrose sedimentation and electron microscopy that three species of R plasmid R100-1 DNA are present in minicells: (a) the composite R plasmid' and its two dissociated parts, (b) the replicon containing the genes for conjugal transfer, (c) the replicon containing the genes for antibiotic resistance (SHEEHY et al., 1973b). The dissociated forms are not found in *E. coli*. The frequency of the small cryptic plasmid in these *S. typhimurium* minicells is increased 3-fold in the presence of R100-1, indicating either that the replication of the cryptic plasmid is stimulated under these conditions or that it segregates more frequently into minicells as they are produced.

3. Hybridization of Minicell DNA to DNA from Plasmid-containing Cells

LEVY (1971a) has presented DNA–DNA hybridization data to prove the identity of R222 DNA extracted from R^+ minicells with the DNA from *E. coli* cells containing the same R plasmid. At least 95% of the DNA isolated from minicells is plasmid DNA by this test. Some degree of homology was indicated by hybridization between the two R plasmids, 222 and N-3. The amount of hybridization of minicell DNA with R^- *E. coli* DNA was only 4%.

4. Recovery of Plasmid Markers from Minicells by Conjugation, Transduction, and Transformation

Biological proof that the DNA in minicells is plasmid DNA has been obtained by conjugation, transduction, and transformation. KASS and YARMOLINSKY (1970) centrifuged F′ *gal*$^+$ containing minicells through a sucrose gradient and tested fractions of the gradient for donor ability. The profile of donor ability paralleled the OD profile for minicells. Purified F′ *gal*$^+$ (*λcI857*)-containing minicells were good conjugal donors of the plasmid marker *gal*$^+$ but, in contrast to F′-containing cells, these minicells did not transfer chromosomal markers. Similar results have been obtained by ROOZEN et al. (1971a) with F′ *lac*$^+$- and F′ KLF-1-containing minicells. Minicells containing either R64-11 or Col-*trp*$^+$ plasmids were also effective conjugal donors of the plasmid, although the frequency of transfer by both cells and minicells was much lower than for F′-containing cells and minicells (ROOZEN et al., 1971a). LEVY and NORMAN (1970) have demonstrated that minicells containing the R plasmids 222 or N-3 have the same transfer efficiency as cell donors. However, some other R plasmids transfer less efficiently with minicells as donors than with cells (LEVY and NORMAN, 1970).

CURTISS (unpubl.) purified minicells from a strain carrying the Col-*trp*$^+$ plasmid by differential centrifugation at 37° C and infected them with P1 transducing phage. Infected minicells were then further purified on a sucrose gradient and 0.3-ml fractions of the gradient were collected into tubes containing 1 ml L broth, incubated 3 h and assayed for P1 plaque forming units and transduction of a *thr*$^-$ *purE*$^-$ *trp*$^-$ strain to Trp$^+$, Thr$^+$, and PurE$^+$. No transductants for the chromosomal markers *thr*$^+$ and *purE*$^+$ were observed while low numbers of Trp$^+$ transductants were found. The profiles for plaque forming units and for Trp$^+$ transducing phage followed the OD profile for minicells from the gradient.

COHEN et al. (1972) and COHEN and CHANG (1973) used R plasmid-containing minicells to prepare various forms of R plasmid DNA to use as transforming DNA in an *E. coli* transformation system. This study demonstrated that all the forms of DNA that can be isolated from minicells (i.e. catenated, CCC, and OC forms) are equally effective in transformation while sonicated or denatured DNA is not effective. It was further shown that the transformants produced by using plasmid DNA from minicells had all of the original genetic properties associated with the presence of the plasmid (drug resistance and/or conjugal fertility).

5. RNA and Protein Species Synthesized in Minicells

Profiles of RNA and protein species synthesized in minicells depend on the plasmid contained (see Sects. IV. G. 2. and 3.) and are distinct from profiles of cellular RNA and protein species, indicating that the RNA and protein synthesized in minicells are plasmid-specific. It has also been shown that most of the RNA synthesized in these minicells will hybridize to plasmid DNA while only 2% of the RNA hybridizes to *E. coli* DNA (VELTKAMP et al., 1974). These are additional proofs that minicells from plasmid-containing strains contain much more plasmid DNA than do random fragments of chromosomal DNA.

B. Mechanism and Frequency of Plasmid Segregation into Minicells

Much evidence has been accumulated to prove that the plasmid DNA found in minicells from plasmid-containing strains gets there by some type of segregation of plasmid DNA into the minicells prior to or during the time of their production, and not by conjugation, transduction, or transformation. Conjugation has been ruled out because: (a) the DNAs from the nonconjugative plasmids ColE1 (INSELBURG, 1970), *λdv* (ROOZEN et al., 1971c), Clo DF13 (KOOL et al., 1972) and 219 (tetracycline-resistant) (VAN EMBDEN and COHEN, 1973) all get into minicells; (b) the amount of plasmid DNA in minicells is about the same for plasmids with both repressed and derepressed expression of conjugal fertility (LEVY, 1971b; ROOZEN, 1971; ROOZEN et al., 1971a); (c) plasmid-containing minicells exhibit entry exclusion, which acts to block conjugal transfer of the same plasmid (COHEN et al., 1967; SHEEHY et al., 1972a, b); (d) minicells that receive single-stranded plasmid DNA by conjugation (COHEN et al., 1968a, b) are unable to synthesize RNA and protein (FRALICK et al., 1969; FRALICK, 1970) and are unable to convert this DNA to circular plasmid molecules (FENWICK and CURTISS, 1973a). Transformation is unlikely since the amount of plasmid DNA in minicells is the same when the culture is grown in either the presence or absence of deoxyribonuclease. Transduction seems an improbable mechanism since most of the minicell-producing strains were not lysogenic for any known transducing phages and, furthermore, some of the plasmids found in minicells contain more DNA than can be carried by known transducing phages.

The plasmids for which segregation into minicells of *E. coli* has been demonstrated are listed in Table 3. Segregation of plasmids into minicells of *S. typhimurium* has also been demonstrated for R100-1, R64-11 and for a cryptic plasmid (SHEEHY et al., 1973a, b). A direct determination of the frequency of minicells that contain plasmid DNA by means of electron-microscopic autoradiography of minicells produced by cultures grown under conditions of steady-state labeling of DNA has not yet been reported. A minimum estimate can be obtained, however, by determining the frequency of minicells that are capable of acting as conjugal donors of derepressed conjugative plasmids. On this basis, F′ *gal* and F′ *gal* (*λ*) plasmids segregate into minicells at a low frequency, and KASS and YARMOLINSKY (1970) estimate that only 1%

Table 3. Types of plasmids segregated into minicells of *E. coli* and a literature survey of papers describing and utilizing these Pla+ minicells

Plasmid[a]	Chromosome[b]	References
F	925, 974	Roozen et al. (1971a), Shull et al. (1971)
F′ KLF-1: F *thr*+ *ara*+ *leu*+	925, 974	Roozen et al. (1971a), Sheehy et al. (1972b)
F′ ORF-207: F *lac*+	925	Roozen et al. (1971a), Fenwick and Curtiss (1973a)
F′14: F *ilv*+ *metE*+ *metB*+ *argE*+ *argH*+	925, P4121	Roozen et al. (1971a), Hori et al. (1974)
F′ KLF-41: F *argG*+ *aroE*+ *argD*+ *aroB*+ *mal*+	P4121	Hori et al. (1974)
F′8: F *gal*+	925	Kass and Yarmolinsky (1970)
F′λ: F *gal*+(λc*I857*)	925	Kass and Yarmolinsky (1970)
R1: R *drd*+ *fi*+ Cm Km Am Sm Su	925, 984	Cohen et al. (1971a, b), Levy and McMurry (1974)
R1-19: R *drd fi*+ Cm Km Am Sm Su	925, 984	Roozen et al. (1971a), Levy (1971a)
R222[c]: R *drd*+ *fi*+ Cm Sm Su Sp Tc	984, 925 *thy*	Levy and Norman (1970), Levy (1971b), Inselburg (1971), Levy and McMurry (1974)
R222[c]-R3: R *drd*+ *fi*+ Cm Sm Su	984	Levy and McMurry (1974)
R100[c]: R *drd*+ *fi*+ Cm Sm Su Sp Tc	925	Sheehy et al. (1972b)
R100-1: R *drd fi*+ Cm Sm Su Sp Tc	925	Roozen et al. (1971a), Sheehy et al. (1972b), Fenwick and Curtiss (1973a)
R6-3*drd*12: R *drd fi*+	925	Cohen et al. (1971a, b)
R64-11: R *drd fi*− Sm Tc	925, 974, 984	Roozen et al. (1971a, b), Levy (1971a), Shull et al. (1971), Sheehy et al. (1972b), Paterson and Roozen (1972b), Cohen et al. (1972), Khachatourians and Riddle (1973), Fenwick and Curtiss (1973a, b, c), Rogerson and Stone (1974)
R64: R *drd*+ *fi*− Sm Tc	984	Levy and McMurry (1974)
R6K: R Am Sm	925, 1411	Macrina and Curtiss (unpublished)
R124: R *fi*+ Tc	984	Levy and McMurry (1974)
R386: R*fi*+ Tc	984	Levy and McMurry (1974)
RM98: R *fi*− Am	984	Levy and McMurry (1974)
CF-2: R Tc	984	Levy and McMurry (1974)
FR1: R Tc	925	Franklin and Foster (1974)
N-3: R *drd*+ *fi*− Sm Su Tc	984	Levy and Norman (1970), Levy (1971a, b), Levy and McMurry (1974)

Table 3 (continued)

Plasmid[a]	Chromosome[b]	References
219: Tc	925	VAN EMBDEN and COHEN (1973)
219: Tc' and I: transfer plasmid	925	VAN EMBDEN and COHEN (1973)
Col-*trp*$^+$: F ColVMB *trp*$^+$	925	ROOZEN et al. (1971a, b), SHULL et al. (1972), SHEEHY et al. (1972b), FRAZER and CURTISS (1973), FENWICK and CURTISS (1973a)
Col-*trpA2*: F ColVB *trpA2*	925	ROOZEN et al. (1971a), FRAZER and CURTISS (1973)
Col V *drd*$^+$	925	ROOZEN et al. (1971a)
Col B *drd*	925	ROOZEN et al. (1971a, b)
Col E1	925, 925 *recA*	INSELBURG (1970, 1972, 1973), INSELBURG and FUKE (1970, 1971)
Col DF13	925	KOOL et al. (1972, 1974), VELTKAMP et al. (1974) FUKE and INSELBURG (1972)
λdv	925 *recA*	ROOZEN et al. (1971c), PATERSON and SETLOW (1972), PATERSON et al. (1973)

[a] Markers encoded by plasmids listed below include *drd*$^+$, repressed donor ability; *drd*, derepressed donor ability; *fi*$^+$, inhibition of transmission of F; *fi*$^-$, no inhibition of transmission of F; Cm, chloramphenicol resistance; Km, kanamycin resistance; Am, ampicillin resistance; Sm, streptomycin resistance; Su, sulphonamide resistance; Sp, spectinomycin resistance; Tc, tetracycline resistance.
[b] Genotypes are given in Table 4, p. 64.
[c] The Plasmid R222 is the same as R100.

of the minicells contain these plasmids. Other F' plasmids (F'*lac* and F'KLF-1) segregate into minicells at a higher level so that at least 15 to 20% of the minicells contain a plasmid (ROOZEN et al., 1971a). Another, more practical way to infer the relative degree of plasmid segregation into minicells has been to determine the percentage of DNA in a culture of cells and minicells that is present in purified minicells. This percentage can vary from 0.08% in F'*gal*- and F'*gal*(*λ*)-containing minicells (KASS and YARMOLINSKY, 1970) to more than 2% for N-3- and R222-containing minicells (LEVY and NORMAN, 1970), and from 0.1 to 4% depending on whether the plasmid is an F, F', R or Col plasmid (ROOZEN et al., 1971a). If these values are used with the known molecular weights for the bacterial chromosome and the plasmid in question, it is then possible to estimate the mean number of plasmid molecules per minicell. Thus the mean number of plasmid molecules per minicell is between 1 and 2 for most R and Col plasmids (ROOZEN et al., 1971a; LEVY, 1971a, b), between 0.5 and 1 for F' *lac*, F' 14 and F' KLF-1 (ROOZEN et al., 1971a), and between 0.1 and

0.3 for F (ROOZEN et al., 1971 a). With regard to F segregation into minicells, it should be noted that initial attempts to demonstrate this were unsuccessful (COHEN et al., 1967). Dimers of the plasmid *λdv* (9×10^6 daltons) segregate into minicells (ROOZEN et al., 1971 c) and since the percentage of the total DNA in the culture found in these minicells is comparable to that found in R plasmid-containing minicells (ROOZEN, 1971; HOUCK and CURTISS, unpubl.), we estimate that there are 8 to 12 *λdv* molecules per minicell. These calculated values for the mean number of plasmids per minicell are not, however, equal to the mean number of plasmids that segregate into minicells at the time of minicell division. Most plasmid-containing minicells are capable of DNA synthesis (see Sect. IV. D.) and thus the amount of labeled DNA present is the sum of the amount segregated into minicells and the amount of new DNA synthesized. It should also be emphasized that not all plasmids can segregate into minicells, e.g. it has not been possible to detect the segregation of either the P1 prophage (KASS and YARMOLINSKY, 1970; ROOZEN, 1971; ROOZEN et al., 1971 a) or the 130×10^6 dalton cryptic plasmid in the *S. typhimurium* minicell producer (SHEEHY et al., 1973 a).

The segregation of F and F′ plasmids into minicells contradicts the concept of strict cosegregation for F and the bacterial chromosome. KASS and YARMOLINSKY (1970) point out that strict cosegregation has been obtained when F′ plasmid replication is inhibited and chromosome replication is not inhibited (CUZIN and JACOB, 1965, 1967; HOHN and KORN, 1969), while independent segregation of F′ plasmids into minicells has been demonstrated under conditions allowing replication of both F′ and the chromosome. Hence, KASS and YARMOLINSKY (1970) suggest that the seeming paradox between strict cosegregation and independent segregation of F′ plasmids might be resolved by determining whether F′ plasmids thermosensitive for replication can segregate into minicells at the nonpermissive temperature.

The mechanism of plasmid segregation into minicells is not known. A high proportion of the plasmid DNA in minicells is membrane-associated as determined by use of the M-band technique (membrane-sarkosyl-magnesium crystals complex) as described by TREMBLAY et al. (1969). LEVY (1971 a) has demonstrated that 60–80% of the DNA from minicells containing R222 (i.e. R100) is associated with the M band. SHULL et al. (1971) found that 93% of R64-11, 78% of Col-*trp*$^+$, and 79% of F DNA in minicells associates with the M band, while HOUCK and CURTISS (unpubl. results) have observed that 84% of R64-11 and 66% of *λdv* DNA in minicells associates with the M band. Thus membrane association could be involved in plasmid segregation into minicells. Additionally, the number of plasmid copies per bacterial cell and the intracellular distribution of plasmids may be extremely important in segregation. It is therefore not clear whether the presence of a plasmid in minicells is due to residence of that plasmid at the extremities of the cell and its subsequent inclusion in minicells, or to replication near the pole of the cell and subsequent distribution to the minicell at the time of formation of the minicell septum.

C. Use of Plasmid-containing Minicells as a Source of Plasmid DNA

The fact that DNA in minicells from plasmid-containing strains is almost exclusively plasmid DNA warrants the use of minicells as an efficient way to purify plasmid DNA, as originally suggested by Roozen et al. (1971a). Indeed, plasmid-containing minicells have been used as a source of purified plasmid DNA for electron microscopy of replicative intermediates including catenated DNA (Cohen et al., 1971a; Fuke and Inselburg, 1972), for transformation experiments (Cohen et al., 1972; Cohen and Chang, 1973), as a reference marker (Nakazawa and Tamada, 1972), and for use in DNA heteroduplex studies (Sharp et al., 1973).

D. Replication of Plasmid DNA in Minicells

1. Incorporation of [^{3}H]Thymidine by Plasmid-Containing Minicells

The capacity of plasmid-containing minicells to incorporate [^{3}H]thymidine into DNA (Roozen et al., 1971a; Roozen, 1971; Levy, 1971a; Veltkamp et al., 1974) suggested that a number of plasmids in minicells could synthesize DNA by replication or repair pathways. Plasmid-containing minicells vary in the extent of [^{3}H]thymidine incorporated, e.g. ColV- and *λdv*-containing minicells incorporate [^{3}H]thymidine to a level two times that of Col-*trp*$^+$- and R64-11-containing minicells (Roozen, 1971). Conclusions about the extent of replication in minicells containing different plasmids are difficult to evaluate on the basis of kinetics of incorporation curves alone, since minicells rapidly convert [^{3}H]thymidine (and probably its analogues) to an uncharacterized compound that is not incorporated into acid-insoluble material (Khachatourians, Roozen, Fenwick, Houck and Curtiss, unpubl.). Thus a better basis for comparing the extent of replication observed with different plasmids is the physical and biochemical characterization of the DNA synthesized by plasmid-containing minicells.

2. Incorporation of [^{3}H]Thymidine into CCC DNA

A significant percentage of the label incorporated into DNA in plasmid-containing minicells is in the CCC DNA fraction characteristic of plasmid DNA. This has been observed consistently with minicells containing ColE1 (Inselburg, 1970, 1973; Inselburg and Fuke, 1971), ColV (Roozen et al., 1971a), R222 (Inselburg, 1971), *λdv* (Roozen et al., 1971c; Roozen, 1971) and Clo DF13 (Veltkamp et al., 1974). It is probably significant that the percentage of DNA observed as CCC DNA, by CsCl–ethidium bromide centrifugation of minicell lysates obtained by a lysozyme-sarkosyl procedure, is higher when label is incorporated by purified minicell suspensions than when label is incorporated by growing cultures. With ColE1, 30 to 60% of the label incorporated by minicell suspensions is found in CCC DNA, while only 15% of the DNA in minicells labeled during growth is in this fraction (Inselburg, 1970).

Similar data are available for ColV, for which 20% of the label incorporated in 30 min by purified minicells is found in CCC DNA, while only 11% is found in this fraction when DNA is labeled before isolating minicells (ROOZEN et al., 1971a). INSELBURG (1970, 1971) has demonstrated that the label found in the CCC DNA fraction defined by CsCl–ethidium bromide or neutral sucrose gradient centrifugation has the characteristics of true CCC DNA. This DNA fraction synthesized by ColE1-containing minicells is rapidly renatured after alkali denaturation and is degraded from the 24S to an 18S form by limited digestion with deoxyribonuclease I (pancreatic) with the same kinetics as for ColE1 CCC DNA (BAZARAL and HELINSKI, 1968). The CCC DNA labeled in R222-containing minicells also is rapidly renatured after alkali denaturation but to a lesser extent than with ColE1 (45% rapidly renatures with R222 while 75% rapidly renatures with ColE1).

3. Proof of Semiconservative DNA Replication in Plasmid-Containing Minicells

Experiments by INSELBURG (1970, 1971) and INSELBURG and FUKE (1971) have demonstrated that the newly synthesized DNA in minicells is probably due to semiconservative replication rather than to a repair process. After labeling in the presence of bromodeoxyuridine, a shift in the density of DNA on CsCl density gradients can be demonstrated to the half-heavy position after 1 hour and to the heavy-heavy position after 3 hours for ColE1-containing minicells (INSELBURG, 1970). A density shift was also demonstrated for R222-containing minicells but only to the half-heavy position after labeling for 2 hours (INSELBURG, 1971). Thus, replicating ColE1 can undergo two rounds of replication in minicells while replicating R222 undergoes only one round of replication. The fraction of plasmid DNA in minicells containing R222 and ColE1 that is capable of replication is not known, and similar density shift experiments with minicells prelabeled before purification would be necessary to determine this point. Acridine orange prevented the shift in density in both ColE1- and R222-containing minicells (INSELBURG, 1971) although, as discussed by CLOWES (1972), the relatively high concentration of acridine orange used (50 μg/ml) is not effective in curing cells of either ColE1 or the R plasmid. ROOZEN (1971) demonstrated that ColV was capable of replicating to a half-heavy density in minicells but *λdv* could not replicate once completely. In reference to all these density shift experiments, it should be noted that the theoretical density shift expected for complete substitution of all thymine by bromouracil was not achieved, even in thymine-requiring minicell-producing strains (ROOZEN, 1971). It is therefore conceivable that there might be some plasmid DNA turnover in minicells with the thymine released being reused for new synthesis, thus reducing the degree of bromouracil substitution. Double-label experiments will therefore be required to investigate this question. Complete proof of semiconservative replication will also require that half-heavy DNA (nicked by deoxyribonuclease) be analysed on alkaline CsCl gradients to show whether each DNA molecule contains one heavy and one light strand.

4. Electron Micrographs of Presumptive Replicating DNA Forms

Experiments using very short periods of labeling in ColE1-containing minicells have demonstrated that labeled material is found in regions of CsCl–ethidium bromide and neutral sucrose gradients where catenated DNA molecules are found. The most label in the catenated fractions was observed when the pulse was 5 sec, i.e. the approximate time required for ColE1 replication and, as the length of the pulse increased, the percentage label in the catenated fractions decreased (INSELBURG and FUKE, 1971). This demonstrated that replicating forms of ColE1 DNA should reside in the catenated DNA portion of the gradient.

Electron microscopy of half-heavy DNA from a density-shift experiment with ColE1-containing minicells revealed that 3% of the plasmid molecules were forked open circles, indicative of replicating DNA. The other DNA structures observed in this study were 8% linear monomers, 29% supercoiled DNA, and 60% open circles (INSELBURG and FUKE, 1970). Electron microscopy of the DNA from the catenated portion of CsCl–ethidium bromide or neutral sucrose gradients has revealed many diverse DNA structures: double-forked monomers of both the open circle and supercoiled types, rolling circle-type structures with the circle either open or supercoiled, catenanes in which one circle of the pair is double-forked, catenanes of dimers that are open-open, twisted (i.e. supercoiled-)open or twisted-twisted, and oligomers of various types including trimer and tetramer catenanes and rolling circles (FUKE and INSELBURG, 1972). Figure 3 shows electron micrographs of representative ColE1 replicative intermediates isolated from minicells by FUKE and INSELBURG (1972). Catenated DNA structures have also been demonstrated by electron microscopy of DNA from R6(3)- and R1-containing minicells taken from the fractions of CsCl–ethidium bromide or neutral sucrose gradients that are characteristic of catenated DNA (COHEN et al., 1971a, b). INSELBURG (1973) has also demonstrated that the catenated DNA observed by electron microscopy is not formed primarily as a result of *rec*-dependent recombination events, since the same percentage of catenanes is observed regardless of whether the ColE1 minicell producer is *recA*$^+$ or *recA*$^-$. Further compelling evidence that catanenes are intermediates in plasmid replication and do not arise by host-controlled recombination events has been obtained by KUPERSZTOCH and HELINSKI (1973) and NOVICK et al. (1973).

5. Conjugal DNA Synthesis in Plasmid-Containing Minicells

Conjugal DNA replication of plasmid DNA evidently occurs in plasmid-containing minicells since minicells can be competent donors of many conjugative plasmids including F′ plasmids (KASS and YARMOLINSKY, 1970; ROOZEN et al., 1971a), certain R plasmids (ROOZEN et al., 1971a; LEVY, 1971a), and Col plasmids (ROOZEN et al., 1971a) as discussed more fully in Section VI. C. LEVY and NORMAN (1970) have observed that the relative transfer from mini-

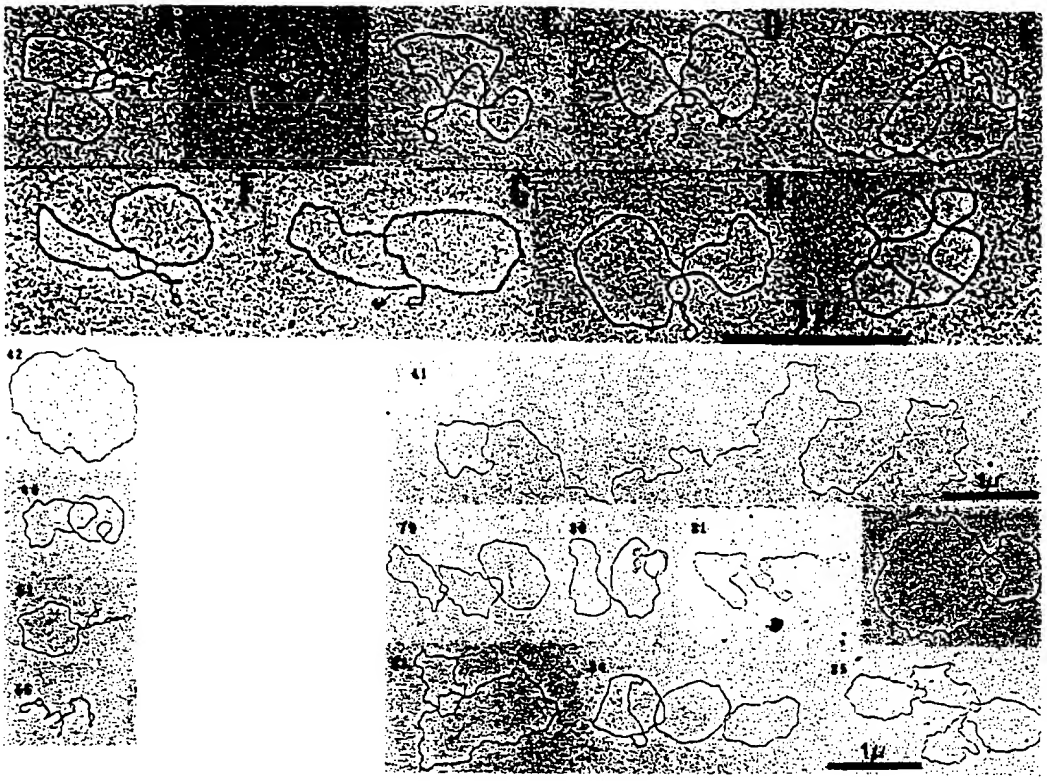

Fig. 3 A—I. Replicative intermediates isolated from ColE1-containing minicells. A—D and F—I show replicating molecules having a twisted unreplicated branch and two untwisted replicating branches. E is a catenated dimer in which one of the molecules is replicating. 42, 46, 51, 56 are four kinds of dimer molecules and 41 a replicating molecule composed of a unit circle and a long double-stranded tail. 79–85 are catenated oligomers including monomers and dimers (82 and 83), a trimer in which one molecule is replicating (84), and a catenated tetramer (85). Markers represent 1 micron; magnification of 42, 46, 51, 56 is the same as for 41. From FUKE and INSELBURG (1972) with permission of the authors and Proceedings of the National Academy of Sciences, U.S.A.

cells of different R plasmids varies, indicating that these plasmids have different capacities to synthesize essential components needed to promote conjugal replication or transfer, or that plasmid-containing strains vary in the distribution of these components to minicells.

6. Potential of Plasmid-Containing Minicells for Studies of Plasmid Replication

The experiments reviewed in this section indicate that minicells may be very useful in studying the vegetative and conjugal replication of some plasmids. The system may not be appropriate for studying large plasmids since it appears that the amount of replication may be limited by plasmid size. Obviously the vegetative replication apparatus is adequate or may be synthesized for the replication in minicells of ColE1, Clo DF13, R222, ColV and

λdv. Replication of the similar plasmids ColE1 (KINGSBURY and HELINSKI, 1970, 1973) and Clo DF13 (VELTKAMP et al., 1974) requires DNA polymerase I, which is present in minicells. Nevertheless, the replication of these plasmids in minicells may differ in some respects from replication of the same plasmid in cells since INSELBURG (1970) has observed that although the presence of chloramphenicol does not inhibit ColE1 replication in minicells, ColE1 replication is not induced by either chloramphenicol or mitomycin C. VELTKAMP et al. (1974) have demonstrated that Clo DF13 replication is slightly stimulated in minicells after inhibition of protein synthesis by either chloramphenicol or puromycin and continues for more than 8 hours. They have also demonstrated that Clo DF13 replication is shut off immediately by addition of rifampicin, which suggested the necessity for an RNA primer for plasmid replication. Indeed, CCC Clo DF13 DNA synthesized in minicells in the presence of chloramphenicol was convertible to the OC form by treatment with either ribonuclease or alkali. Clo DF13 DNA replicated in minicells in the absence of chloramphenicol is not converted from the CCC to OC form by these treatments; thus this RNA primer must be removed from plasmid molecules during normal replication.

Many relevant aspects of plasmid replication have not yet been explored in depth with the minicell system. For instance, plasmid DNA is apparently associated with the minicell membrane (FRALICK, 1970; LEVY, 1971a; SHULL et al., 1971; HOUCK and CURTISS, unpublished) as discussed in Section IV. B.; however, a correlation between membrane association and the replication of plasmids is yet to be established in minicells. An important area that should be investigated more thoroughly is the presence or absence of relaxation complexes (CLEWELL and HELINSKI, 1970, 1972) in minicells. The minicell lysis and/or centrifugation techniques for DNA which have been employed (except for one instance, see below) are known to destroy the relaxation complex of ColE1, thereby decreasing the potential yield of CCC DNA. The variability in the yield of CCC DNA observed with various techniques may be due to specific loss of some intermediate, like a relaxation complex, in the plasmid replication cycle. Examples of this variability in yield of CCC DNA are either 15 or 80% CCC DNA for ColE1 on CsCl–ethidium bromide or neutral sucrose sedimentation gradients, respectively (lysozyme-sarkosyl lysis) (INSELBURG, 1970), and either 19 or 47% CCC DNA for *λdv* on neutral sucrose sedimentation with detergent lysis by sodium dodecyl sulfate (SDS) or Triton X-100, respectively (ROOZEN, 1971). This last example is particularly intriguing since lysis with the neutral detergent Triton X-100 and centrifugation on a neutral sucrose gradient should preserve a relaxation complex and this is the only instance of a lysis centrifugation procedure for DNA from plasmid-containing minicells where this statement can be made. Thus a significant amount of *λdv* DNA may be in a relaxation complex in minicells, making this a potentially useful system for testing the role of relaxation complexes in plasmid replication. If the relaxation protein is indeed present, then there is a good chance that it is encoded by one of the 5 or 6 genes on the *λdv* genome.

E. Repair of Plasmid DNA in Minicells after UV- and Ionizing-Irradiation

Considerable work has been done on the DNA repair capabilities of minicells and the contribution of the *recA* system to these repair phenomena. At the outset it is well to note that the original minicell producer χ925 was discovered among ionizing (i.e. X-ray) radiation-resistant mutants (ADLER et al., 1967; ADLER and HARDIGREE, 1972). The relationship of ionizing radiation-resistance to minicell production is not yet known (see Sect. II. B.). The studies discussed here have been done with derivatives of χ925, which consequently show increased X- and γ-irradiation resistance compared to the non-minicell-producing parent strain, P678 (PATERSON and ROOZEN, 1972b). Strain χ925 has wild-type sensitivity to UV-irradiation; the presence of the R64-11 plasmid in the χ925 background confers on the strain increased UV-resistance. The production of UV- and γ-irradiation-induced damage in DNA is the same in minicells and cells (PATERSON and ROOZEN, 1972b).

Both R64-11 and *λdv*-containing minicells have been examined for repair of UV-, X- and γ-irradiation damage by ROOZEN et al. (1971d), PATERSON (1972), ROOZEN (1971), PATERSON and ROOZEN (1972a, b), PATERSON and SETLOW (1972), and PATERSON et al. (1972, 1973). In these studies cultures were labeled with [^{3}H-methyl]thymidine and exposed to UV-, X- or ^{60}Co γ-irradiation under various conditions. Cultures were then incubated in growth medium for different time intervals to allow for repair before purifying cells and minicells by sucrose gradient centrifugation. Cells and minicells were then lysed and the DNA damage analyzed by the loss of CCC DNA recovered by velocity sedimentation in neutral and alkaline sucrose gradients. Minicells that were purified before irradiation had exactly the same repair capabilities as minicells not exposed to sucrose. This was also true for minicells exposed to 20% sucrose for as long as cells were exposed during purification (PATERSON and ROOZEN, 1972b). Cells, however, that were exposed to sucrose before irradiation had a greatly reduced capacity to repair γ-irradiation-induced single-strand breaks as well as a reduced capacity to excise UV-induced thymine dimers (PATERSON and ROOZEN, 1972b). However, sucrose treatment of cells did not inhibit photorepair of UV-induced damage. These results suggest that sucrose treatment may affect the function of DNA polymerase I or of DNA ligase or some other repair enzyme in cells while having little or no effect on these activities in minicells.

The repair of thymine-containing dimers induced by UV-irradiation has been examined in minicells containing the plasmid R64-11 (PATERSON and ROOZEN, 1972b). Cells were twice as effective as plasmid-containing minicells in photoreactivation repair, which is of particular note since minicells without a plasmid have no photoreactivation enzyme activity (SETLOW and COHEN, cited in COHEN et al., 1968b). No excision repair occurred in R64-11-containing minicells, presumably due to the low activity of UV endonuclease.

The γ-irradiation-induced damage to DNA and its repair has been studied with R64-11- (PATERSON and ROOZEN, 1972b) and *λdv*-containing minicells

(PATERSON and SETLOW, 1972; PATERSON et al., 1972, 1973) (which are from a minicell producer that is also *recA*$^-$). It was observed that three different types of lesions or alterations in DNA were induced by γ-irradiation in plasmid-containing minicells: (a) single-strand breaks in the sugar-phosphate backbone of DNA; (b) alterations resulting in single-strand breaks only upon exposure of the DNA to an alkaline pH, i.e. alkaline-labile bonds (PATERSON and ROOZEN, 1972a); (c) damage which makes the DNA susceptible to the attack of an endonuclease purified from *Micrococcus luteus*. This third type of lesion had not been previously described, and the endonuclease activity from *M. luteus* has not been completely characterized (PATERSON and SETLOW, 1972). It was further observed that the endonuclease activity of a partially purified *M. luteus* extract was depressed upon addition of UV-irradiated calf-thymus DNA. Thus PATERSON and SETLOW (1972) suggested that the same *M. luteus* endonuclease might recognize and attack both types of damage (i.e. UV- and γ-irradiation-induced lesions). Irradiation in air or in a N_2 atmosphere affected the frequency of each type of γ-irradiation-induced lesion differently: (a) the production of single-strand breaks, measured on alkaline sucrose gradients, was 3 to 4 times greater in air (PATERSON et al., 1973); (b) the frequency among all strand breaks of alkaline-labile bonds was about 2 times greater under anoxic conditions (PATERSON et al., 1973); (c) the presence of *M. luteus* endonuclease-susceptible lesions was essentially the same under aerobic or anoxic conditions (PATERSON and SETLOW, 1972).

The repair of γ-irradiation-induced single-strand breaks was fairly rapid and complete in R64-11- and *λdv*-containing minicells (PATERSON and ROOZEN, 1972b; PATERSON et al., 1972, 1973). In addition, repair of single-strand breaks induced by X-irradiation has also been observed in plasmid-containing minicells (ROOZEN et al., 1971b; CRELLIN PAULING, pers. comm.). As pointed out by TOWN et al. (1971), there are at least two mechanisms for repair of γ-irradiation-induced single-strand breaks: (a) rapid repair probably involving DNA polymerase I; (b) slow repair involving the *recA*, *recB* and *recC* gene products. In order to test the first part of this hypothesis, it would be very interesting to determine whether the rapid type of repair in plasmid-containing minicells occurs in a *polA*$^-$ (i.e. DNA polymerase I-deficient) strain. The slow repair of single-strand breaks, which is also observed in minicells, is interesting since it occurs equally in minicells derived from *recA*$^+$ and *recA*$^-$ strains. The mechanism of this type of repair should be investigated in more detail. The γ-irradiation-induced alkaline-labile bonds were not repaired at all in *λdv*-containing minicells, which may suggest that this type of DNA alteration is not biologically harmful to cells (PATERSON et al., 1973). The *M. luteus* endonuclease-susceptible lesions were rapidly repaired in *λdv*-containing minicells (PATERSON and SETLOW, 1972).

The relative capacities of cells versus minicells to perform various types of repair of radiation-induced damage to plasmid DNA provides further information about enzyme distribution in cells. For instance, the UV endonuclease and photoreactivation enzyme may be preferentially localized in the central portions

rather than the polar ends of cells (PATERSON and ROOZEN, 1971), or they may be present in very few molecules per cell, which would explain the lower activity of these enzymes in minicells. The photoreactivation enzyme, but not the UV endonuclease, may segregate to minicells with plasmid DNA. It is possible that these enzymes, particularly the UV-endonuclease, are preferentially bound to chromosomal rather than plasmid DNA. A possible mechanism to explain such preferential binding might be that the amount of enzyme bound is proportional to DNA length. By use of plasmid-containing minicell-producing strains that differ with regard to either the size or amount of plasmid DNA segregated into minicells, it should be possible to reach more definite conclusions about the various enzymes involved in repair and/or DNA metabolism and their subcellular distribution.

F. Use of Plasmid-Containing Minicells to Study Deoxyribonuclease Activities

Minicells containing R64-11 have been used to examine colicin E2-induced degradation of DNA by observing the nicking of CCC DNA and its degradation to acid-soluble material (KHACHATOURIANS and RIDDLE, 1973). The kinetics of loss of acid-insoluble material was the same in cells and minicells, indicating that the nucleases involved have similar activities in cells and minicells. Minicells do contain exo- and endonuclease activities, even in an *endI*$^-$ minicell producer, that attack single-stranded DNA transferred to minicell recipients in conjugation (KHACHATOURIANS et al., 1972, 1974). Whether any of these nuclease activities are related to the colicin E2-induced nuclease activities is yet to be determined. PATERSON and VAN DORP (in prepn, 1974) have recently found that the concentration of the ATP-dependent exonuclease V that is specified by the *recB* and *recC* genes is present in approximately equal amounts in DNA-deficient *recA*$^+$ minicells from χ925, in λdv-containing *recA*$^-$ minicells and in R64-11-containing *recA*$^+$ minicells.

G. Synthesis of Plasmid-Specific Gene Products in Minicells

1. Incorporation of Radioactive Precursors into RNA and Protein by Plasmid-Containing Minicells

Plasmid-containing minicells can incorporate radioactive precursors for RNA synthesis, i.e uracil and uridine (FENWICK et al., 1970; ROOZEN et al., 1971 a, b; LEVY, 1971 a; VELTKAMP et al., 1974; KOOL et al., 1974; HORI et al., 1974) and for protein synthesis (FENWICK et al., 1970; LEVY, 1971 a, b; ROOZEN et al., 1971 b; COHEN et al., 1971 b; FRAZER and CURTISS, 1972; KOOL et al., 1972, 1974; VAN EMBDEN and COHEN, 1973; VELTKAMP et al., 1974; FRANKLIN and FOSTER, 1974; LEVY and MCMURRY, 1974) into acid-insoluble material. In general, the rate of incorporation of precursors into RNA and protein by minicells is only 1 to 2% of the rate observed with a cell suspension of the same OD. It has been demonstrated by electron microscopy and autoradiography that

this incorporation of precursors into RNA and protein is performed by purified minicells and is not due to cells contaminating the minicell preparation (ROOZEN et al., 1971 b). Different plasmid-containing minicells have different capacities for the incorporation of precursors into RNA and protein. ROOZEN et al. (1971 b) have observed that the incorporation of F- and some F'-containing minicells is much lower than that of R^+ or Col^+ minicells. The relatively poor performance of the F- and F'-containing minicells must be partly due to the lower frequency of segregation of these plasmids into minicells (Sect. IV. B.), but other factors may be operating as well.

VAN EMBDEN and COHEN (1973) have studied incorporation of [^{3}H]leucine by minicells containing the small (i.e. 5.8×10^6 dalton) 219 plasmid, which encodes tetracycline resistance, and have demonstrated that: (a) minicells produced by a culture grown in the presence of tetracycline have a faster rate and greater extent of protein and RNA synthesis, probably due in part to the 64% increase in DNA segregated to minicells under these conditions; (b) protein and RNA synthesis in minicells, produced by a culture grown in the absence of drug, are stimulated by addition of tetracycline to the incorporation medium; (c) the small amount of protein synthesis in DNA-deficient minicells is inhibited by tetracycline. These exciting results indicate that the regulation of protein synthesis in plasmid 219-containing minicells is uniquely affected (i.e. stimulated) by the presence of tetracycline. VAN EMBDEN and COHEN (1973) have also examined the proteins synthesized in 219-containing minicells by polyacrylamide-gel electrophoresis (see Sect. IV. G. 3.).

In contrast to the results of VAN EMBDEN and COHEN (1973), FRANKLIN and FOSTER (1974) have observed that protein synthesis in purified minicells containing the FR1 plasmid (which endows the host cell with resistance to tetracycline but carries no other drug-resistance markers) is inhibited almost completely by the presence of tetracycline. Furthermore, protein synthesis by FR1-containing minicells, obtained from cultures grown in the presence of tetracyline (i.e. under conditions where resistance to tetracycline is induced in cells), was also inhibited in the presence of tetracycline, but not as drastically as in minicells obtained from uninduced cultures. A slight increase in resistance to tetracycline was observed when a previously uninduced suspension of purified minicells was incubated with a low level of tetracycline (1 μg/ml) for one hour. At present there is no rationale to explain the difference in expression of these two R plasmids (i.e. 219 and FR1) in minicells.

As discussed in more detail in Section IV. G. 3., expression in minicells of R plasmids encoding tetracycline resistance has also been studied by LEVY and MCMURRY (1974). They have clearly demonstrated the synthesis of a specific polypeptide after exposing purified minicells to 10–15 μg/ml of tetracycline. It would be useful in comparing and contrasting the work of LEVY and MCMURRAY (1974) with that of VAN EMBDEN and COHEN (1973) and FRANKLIN and FOSTER (1974) to know the effect of tetracycline on overall protein synthesis in minicells containing diverse R plasmids encoding tetracycline resistance. Unfortunately, LEVY and MCMURRAY (1974) have not reported such data; how-

ever, they have observed that 10% of the protein synthesized in R222 (i.e. R100)-containing minicells is found in the tetracycline-induced polypeptide.

The effects of various inhibitors of RNA and protein synthesis on incorporation has been tested. Incorporation of [^{3}H]uridine is inhibited by rifampin (100 μg/ml), an inhibitor of initiation of RNA synthesis, in R64-11- and Col-*trp*$^+$-containing minicells (ROOZEN et al., 1971 b) as is the incorporation of [^{3}H]uracil by R222 (i.e. R100)-containing minicells (LEVY, 1971 a). VELTKAMP et al. (1974) have also reported that [^{3}H]uridine incorporation is inhibited by rifampin (100 μg/ml) in Clo DF13-containing minicells. Protein synthesis is inhibited by rifampin in R64-11- (ROOZEN et al., 1971 b; LEVY, 1971 a) and R222-containing minicells (LEVY, 1971 a). However, VELTKAMP et al. (1974) have reported that in spite of the fact that rifampin rapidly inhibits RNA and DNA synthesis in Clo DF13-containing minicells, protein synthesis continues in the presence of the drug. This observation suggests that stable mRNA is present in Clo DF13-containing minicells under physiological conditions. Chloramphenicol inhibits protein synthesis in Col-*trp*$^+$- (ROOZEN et al., 1971 a), R64-11- (ROOZEN et al., 1971 b; LEVY, 1971 a) and Clo DF13-containing minicells (VELTKAMP et al., 1974) but not in R222-containing minicells (LEVY, 1971 a), which indicates that the chloramphenicol-acetylating enzyme encoded by R222 is present in these minicells.

Minicells that have no plasmid exhibit a small amount of incorporation of precursors into protein (ROOZEN et al., 1971 b; COHEN et al., 1971 b; LEVY, 1971 a; KOOL et al., 1974; FRANKLIN and FOSTER, 1974) and into RNA (KOOL et al., 1974; HORI et al., 1974). Protein synthesized in these DNA-deficient minicells has been seen as a small radioactive peak (COHEN et al., 1971 b) or as random high background (KOOL et al., 1974) on SDS polyacrylamide-gel electrophoresis. LEVY (1971 a) reported that the small amount of protein synthesized by DNA-deficient minicells purified at 23° C is greatly reduced if minicells are prepared at 0° C. The low level of protein synthesis in DNA-deficient minicells may be due to persistence of stable cellular mRNA. Alternatively, the low level of protein and RNA synthesis may be due to transcription and translation specific for the small amount of random cellular DNA that is trapped in these minicells.

2. RNA Species Synthesized in Plasmid-Containing Minicells

Some of the RNA products synthesized in minicells containing R64-11, Col-*trp*$^+$ or ColB*drd* were examined on polyacrylamide-disc-gel electrophoresis and by neutral sucrose velocity sedimentation (FENWICK et al., 1970, 1971; ROOZEN et al., 1971 b). In these studies, RNA of heterogeneous molecular weight (i.e. about 5 to 16S) was observed with Col plasmids, and at least four distinct peaks with mobilities from 6 to 9S were observed with the R64-11 plasmid. RNA of lower molecular weight migrating at about 3.8S was observed with all three plasmids and in addition RNA migrating at about 4.6S was observed with the Col plasmids. The physiological role of these various RNA species is not at all

clear and much more work is required to demonstrate the number of species present and to characterize them. JARRY has observed (pers. comm., 1973) that low-molecular-weight RNA of approximately 4S and 7S is synthesized by some F'-containing minicells, but fingerprints indicate that these are not homogeneous RNA species. In hybridization experiments using ϕ80 *pt190h* bacteriophage DNA and total ^{3}H-labeled RNA from Col-*trp*$^+$-containing minicells, the presence of *trp* operon mRNA was demonstrated (FENWICK et al., 1971; ROOZEN, 1971).

NIJKAMP et al. (1973) and KOOL et al. (1974) have identified putative mRNA species synthesized by Clo DF13-containing minicells, which could account for 85% of the coding capacity of the plasmid genome. One RNA species of 21.3S was observed to be synthesized by minicells containing the wild-type Clo DF13 plasmid, and this size of mRNA would be adequate to code for the three plasmid-specific polypeptides synthesized under these conditions. When a mutant plasmid that is present at a level of 40 rather than 15 plasmid copies per cell was studied, the synthesis of three additional RNA species of 19.5S, 14.0S and 12.0S was observed. In addition, KOOL et al. (1974) have done DNA-RNA hybridization experiments to demonstrate that the RNA synthesized by Clo DF13-containing minicells is specific for Clo DF13 plasmid DNA and that only 2.0% of the RNA synthesized in minicells hybridizes to the *E. coli* chromosome. Presumably this *E. coli*-specific RNA was synthesized from random chromosomal DNA fragments present in minicells (KOOL et al., 1974).

HORI et al. (1974) have clearly demonstrated that *E. coli* minicells containing the F'14 plasmid (which encodes one of the *E. coli* ribosomal RNA cistrons) synthesize ribosomal RNA. The ribosomal RNA synthesized in F'14-containing minicells could be isolated in particles of 25S and 18S that are probably similar to the particles of protein and nascent ribosomal RNA that have been observed in cells when the synthesis of ribosomal proteins is blocked by chloramphenicol while ribosomal RNA synthesis continues. HORI et al. (1974) have demonstrated that the 23S and 17S RNA species synthesized by minicells are effectively competed by cellular 23S and 16S ribosomal RNA in DNA-RNA competition hybridization experiments. It is not clear from the data whether 5S ribosomal RNA was also synthesized by F'14-containing minicells. As pointed out by HORI et al. (1974), their experiments indicate that minicells lack an appreciable pool of ribosomal protein, and the data further demonstrate that concomitant expression of an operon for ribosomal protein is not essential for transcription of a ribosomal RNA operon. This interesting work further suggests that the F'14-containing minicells could be used to determine whether any ribosomal protein genes are encoded on the F'14 plasmid and whether stringent control of RNA synthesis operates in minicells.

Recently, MORROW et al. (1974) have examined the synthesis in minicells of eukaryotic rRNA specified by rRNA genes coupled to *E. coli* plasmid DNA.

3. Protein Species Synthesized in Plasmid-Containing Minicells

Meny investigators have examined the proteins synthesized in plasmid-containing minicells. The proteins synthesized by R6-3*drd*12-containing minicells displayed about 14 bands when examined by polyacrylamide–ethylene diacrylate SDS disc-gel electrophoresis (COHEN et al., 1971 b). Proteins synthesized by R222 (i.e. R100)-containing minicells showed label in 14 to 15 bands when subjected to polyacrylamide disc-gel electrophoresis in the absence of SDS (LEVY, 1971 a, b). LEVY (1973) has further separated these labeled proteins into membrane and soluble fractions by differential centrifugation of sonicated minicell extracts. Electrophoresis of membrane proteins on SDS polyacrylamide disc gels reveals 5 to 8 protein bands, and the pattern of membrane proteins shows that some components are specific for different R plasmid mating types; one major component was synthesized by all the R plasmids examined. Of the soluble proteins synthesized by R plasmid-containing minicells, approximately 2% have some affinity for DNA of various types as demonstrated by affinity chromatography. The function of these proteins is under investigation (LEVY, 1973).

KOOL et al. (1972, 1974) have examined polypeptides synthesized by minicells containing the small Clo DF13 plasmid (6×10^6 daltons). The synthesis of three polypeptides of 70000, 20000, and 11000 daltons was observed with the wild-type plasmid while the synthesis of five additional polypeptides of 58000, 44000, 28000, 16000 and 14000 daltons was detected with a mutant plasmid which is characterized by a higher copy number per cell. These 8 polypeptides account for 85% of the coding capacity of the mRNA species synthesized by these minicells. The function of all these polypeptides has not been determined but the 58000-dalton species is close to the size expected for the cloacin protein.

VAN EMBDEN and COHEN (1973) using another small plasmid, i.e. the 219 plasmid (5.8×10^6 daltons) encoding tetracycline resistance, incubated plasmid 219-containing minicells for 3 h in [^{3}H]- or [^{14}C]leucine, extracted minicells, and electrophoresed the polypeptides in SDS polyacrylamide gels. The results clearly showed that 5 polypeptides are synthesized in these minicells and furthermore that the relative amount of synthesis for 4 of the polypeptides is significantly altered by incorporation in the presence of tetracycline (5 μg/ml) or by growth of cultures in the presence of the drug (12.5 μg/ml) prior to isolation of minicells. This is further evidence that protein synthesis in these minicells is regulated by tetracycline.

LEVY and MCMURRAY (1974) examined the proteins synthesized in minicells containing 9 different R plasmids (see Table 3) by SDS polyacrylamide-gel electrophoresis. The synthesis of a tetracycline-inducible (10–15 μg drug/ml) polypeptide was observed only in minicells containing R plasmids specifying tetracycline resistance (i.e. R222, R124, R386, R64, N-3, and CF-2). None of the R plasmids that do not specify resistance to tetracycline synthesized this protein (i.e. R222-R3, R1, and RM98). It was demonstrated that this protein

was enriched in the membrane fraction of minicell sonicates and, in the case of R222, it was estimated that 70% of the protein was membrane-associated. It is significant that identification of the tetracycline-inducible membrane protein was impossible in sonicates of induced cells due to the large number of other membrane proteins synthesized by cells. Thus it is evident from the work of VAN EMBDEN and COHEN (1973) and of LEVY and MCMURRAY (1974) that minicells should be very useful in further characterizing the protein components responsible for tetracycline resistance and their mechanism of action, at least for a large number of R plasmid-host cell systems (see Sect. IV. G. 1.).

4. Synthesis of Functional Protein in Plasmid-Containing Minicells

Three kinds of observations with plasmid-containing minicells give credence to the idea that these minicells are competent to synthesize functional protein.

1. Bacteriophages T4 and P1 can productively infect minicells from plasmid-containing strains (see Fig. 4) although the burst sizes observed are small (see Sect. V.).

2. It can be inferred from some observations by LEVY (1971 b) that minicells synthesize proteins that are required for donor ability since conjugation with R plasmid-containing minicell donors is inhibited considerably either by amino acid starvation prior to mating or by overnight refrigeration; incubation of these plasmid-containing minicells in broth partially restores donor ability. This is especially interesting since F′-containing minicells are effective conjugal donors but seem to have a reduced capacity for RNA and protein synthesis and contain DNA that is not readily isolated as CCC DNA.

3. HAMKALO and FRAZER (unpubl., cited in FRAZER and CURTISS, 1973) have demonstrated by electron microscopy that polysomes are present in Col-*trpA2*-containing minicells (see Fig. 5) and it has been observed that the proportion of actively transcribing minicells is rather high. The minicell-producing strain (χ1298, a *dapD*$^-$ derivative of χ984, described in Table 4 and Appdx II. B. 4., containing the Col-*trpA2* plasmid) was grown to mid- or late-log phase either in L broth containing 0.5*M* sucrose, 50 μg α,ε-diaminopimelic acid/ml and 88 μg L-lysine/ml or in ML salts containing 0.5% glucose, 1.5% Casamino acids, 75 μg α,ε-diaminopimelic acid/ml, 88 μg L-lysine/ml, and other nutritional requirements. Cultures were lysed by incubation with 50 μg T4 lysozyme/ml for 15 sec at 37° C followed by dilution (about 50-fold) and vigorous mixing in deionized water at pH 9. A small amount of formaldehyde in sucrose was added to the lysate, which was centrifuged at about 12000 × *g* for 2 min at 4° C to remove some cells from the preparation and samples from the supernatant were subsequently deposited onto carbon-coated electron microscope grids for staining by phosphotungstic acid, as described by HAMKALO and MILLER (1973). Previous attempts to obtain extrusion of the DNA and polysomes from plasmid-containing minicells (*dap*$^+$) were unsuccessful (HAMKALO, FENWICK and CURTISS, unpubl.), which suggests that the *dapD*$^-$ mutation in strain χ1298 results

Fig. 4. T4 bacteriophage attached to *E. coli* minicells. ×58 500. Electron micrograph taken by D. P. ALLISON

in the formation of minicells that are easier to lyse. It is evident from Fig. 5 that the osmotic shock lysis, though reasonably effective, was less than optimal since only a portion of the extruded contents from the minicell are well spread out on the grid, and most of the ribosomes appear to be missing the 50S subunit. Nevertheless, a polysome that is at least 40 ribosomes long is clearly evident in Fig. 5 and this length is sufficient to accommodate polycistronic mRNA (HAMKALO and MILLER, 1973).

It has been demonstrated that minicells can synthesize a specific functional protein (FRAZER and CURTISS, 1973). Purified minicells containing Col-*trp*$^+$ or Col-*trpA2* when starved for tryptophan rapidly became derepressed for the plasmid-encoded tryptophan operon, as demonstrated by a 1.6- to 3-fold increase after 30 min of derepression in the level of the first enzyme in the tryptophan biosynthetic pathway, anthranilate synthase; DNA-deficient minicells could not be derepressed for enzyme synthesis. It should be mentioned that the activity of anthranilate synthase is dependent upon its binding to the second enzyme in the tryptophan pathway. Thus these results indirectly demonstrate

Fig. 5. The extruded contents of an osmotically shocked *E. coli* Col-*trpA2*$^-$-containing minicell. Arrow indicates one polysome that is about 40 ribosomes long. ×40080. Electron micrograph taken by B. A. HAMKALO

the synthesis of functional phosphoribosyl anthranilate transferase. The ratio of anthranilate synthase synthesized per minicell to the amount synthesized per cell was 1:20. However, when expressed on the basis of amount of enzyme synthesized per unit volume, the ratio of synthetic capacity for minicells compared to cells was 1:2.4. Thus, the synthetic capacity in minicells and cells is fairly similar. Derepression was rapidly blocked in the presence of chloramphenicol (200 μg/ml) and inhibited by rifamycin SV (280 μg/ml) but not until at least 6 min after addition of rifamycin SV. Thus *de novo* synthesis of an-

thranilate synthase and of mRNA was required for the increase in enzyme activity. Incubation of minicells in the presence of tryptophan, the corepressor of the *trp* operon, also blocked enzyme synthesis. Hence regulation of *trp* operon expression is essentially the same in minicells as in cells and the various regulatory components for the *trp* operon (e.g. repressor protein) must be fairly stable in minicells. Evidence has also been obtained by LEVY (pers. comm., 1973) that minicells containing the R222 (i.e. R100) plasmid synthesize the streptomycin-adenylating enzyme and that the synthesis of the enzyme continues for at least 3 h.

5. Potential Usefulness of Plasmid-Containing Minicells in Studying Plasmid-Specific Transcription and Translation

It must be emphasized that not all model enzyme induction or derepression systems function in minicells. For instance, we have been unable to demonstrate induction of β-galactosidase in purified minicells containing the F'*lac* (ORF-207) plasmid (FRAZER, unpublished) and thermal induction of λ phage could not be demonstrated with F'*gal* (*λcI857*)-containing minicells by KASS and YARMOLINSKY (1970). Minicells containing the Clo DF13 plasmid are not inducible for cloacin synthesis after treatment with mitomycin C although cells are inducible (KOOL et al., 1972, 1974). In addition, although the synthesis of anthranilate synthase can be derepressed in minicells containing the Col-*trp*$^+$ and Col-*trpA2* plasmids, enzyme synthesis cannot be "induced" by treatment with indole-3-propionic acid (FRAZER and CURTIS, 1973). The reason(s) for these failures is at present elusive. Consequently, the usefulness of plasmid-containing minicells to investigate plasmid-specific transcription and translation products or the regulation of synthesis of such products may be limited. Obviously the possible interaction between plasmid encoded components and chromosomal encoded components active in promoting the expression of plasmid functions may be quite complex. Much variability among plasmids with respect to expression of specific plasmid genes in minicells must be expected. Protease or ribonuclease activity against specific regulatory proteins or RNA species might contribute to the complexity in patterns of plasmid expression. It has been reported that certain protease activities specifically degrade some regulatory proteins, e.g. the *araC* gene product (YANG and ZUBAY, 1973). No information is available on the proteases present in minicells and only limited information on specific ribonucleases. This is a critical deficiency when attempting to interpret observations on the synthetic capabilities of plasmid-containing minicells for specific gene products as well as for total protein and RNA synthesis. For instance, better rates of incorporation into RNA and protein are observed with minicells harvested from stationary-phase rather than log-phase cultures (ROOZEN et al., 1971 b). Also extracts of DNA-deficient minicells from stationary-phase cultures are more efficient in supporting *in vivo* translation of artificial mRNA than minicells from log-phase cultures, possibly due to lower ribonuclease activity in minicells from stationary-phase cultures (FRALICK et al., 1969; DVORAK et al.,

1970). Little work has been done on turnover of mRNA or other plasmid-specific gene products in minicells.

V. Infection of Minicells with Bacteriophages

Early attempts to infect DNA-deficient minicells from *E. coli* with a variety of phages demonstrated that these minicells were unable to yield progeny phage. ADLER et al. (1967) were able to demonstrate absorption and lysis by phage T6 to these minicells and CURTISS (unpublished) demonstrated lysis from without by using UV-irradiated T4 and T6 or by T4 and T6 ghosts. T3 (ADLER et al., 1967; CURTISS, unpublished) and λ (KELLENBERGER-GUJER, unpublished) seemed to be unable to absorb and/or to inject their DNA into DNA-deficient minicells, which suggests that attachment of these phages with subsequent injection of phage DNA may require cellular activities that are not supplied by minicells and that may be unnecessary for phages with a contractile tail sheath that facilitates DNA penetration into the cell.

FRALICK (1970) demonstrated that T4-infected minicells were unable to incorporate radioactive amino acids, uridine, or thymidine into macromolecules. Seventy to 99% of the T4 phages adsorbed and injected their DNA, as shown by conventional methods as well as by measuring the frequency of collapsed T4 phage attached to minicells (see Fig. 4) and the percentage of labeled phage DNA sedimentable with the minicells. SHULL et al. (1971) were also able to show that 65% of the labeled T4 DNA became associated with the minicell membrane as detected by the M-band technique. VALLÉE et al. (1972) demonstrated that the attachment of T4 ghosts to DNA-deficient minicells also altered the physiological activities of minicells, as evidenced by the almost complete inhibition of leucine transport.

The discovery of plasmid-containing minicells permitted further studies on phage infection. ROOZEN et al. (1971b) were able to infect about 0.1% of sucrose gradient-purified minicells containing either the Col-*trp* or R64-11 plasmids with T4. Burst sizes of these infected minicells varied from 9 to 36. The low infectivities and yields may be explained in part by the observation that exposing cells to sucrose prior to phage infection reduced both infective centers and burst size by 60 to 80%. As further proof that infected minicells were responsible for the T4 phage produced, ROOZEN et al. (1971b) demonstrated that the optical density profile of minicells on 5 to 20% linear sucrose gradients superimposed the infective center profile. As previously mentioned (see Sect. IV. A. 4.), CURTISS (unpubl.) was able to infect Col-*trp*-containing minicells with phage P1 and obtained P1 transducing phages that were able to transduce *trp*$^-$ strains to Trp$^+$.

KASS and YARMOLINSKY (1970) were unable to get the *λcI857* temperature-inducible prophage to excise and/or replicate to produce infectious phage in minicells containing the F′ *gal*$^+$ (λcI857) plasmid. Since minicells can replicate the plasmid *λdv* (ROOZEN et al., 1971c), it is likely that some aspect of excision of λ prophage is blocked. In this regard, it should also be reiterated that F- and

Fig. 6. An F′ *lac*-containing *E. coli* minicell bearing two donor pili that are demonstrated by lateral attachment of RNA donor-specific bacteriophage to both pili and the terminal attachment of a DNA donor-specific bacteriophage to one of the pili. ×43830. Electron micrograph taken by D. P. ALLISON

F′-containing minicells are not very efficient at RNA and protein synthesis as compared to R and Col plasmid-containing minicells (ROOZEN et al., 1971 b). Attempts to productively infect plasmid-containing minicells that possess donor pili with various donor-specific phages have also been unsuccessful (HOFSCHNEIDER, pers. comm.; PRATT, pers. comm.), although the adsorption of RNA and DNA donor-specific phages has been demonstrated by electron microscopy (see Fig. 6).

REEVE and MENDELSON (1973 b) have found that the phages SP01, SP17 and ϕ29 all rapidly adsorb irreversibly to *B. subtilis* minicells but fail to produce any infectious progeny phage. CORNETT and REEVE (1974) extended these studies to more critically examine the step at which SP01 infection of *B. subtilis* minicells was blocked. They initially demonstrated that ^{3}H-labeled SP01 DNA was injected into minicells and was not degraded to acid-soluble material

during 3 h of incubation. These infected minicells, unlike the phage-infected DNA-deficient minicells of *E. coli*, were able to incorporate uridine into RNA and amino acids into protein for an extended period of time. Such syntheses were blocked by rifampin and chloramphenicol, respectively. In spite of the ability of these minicells to synthesize RNA and protein, they were unable to synthesize new SP01 DNA (CORNETT and REEVE, 1974). These results imply that *B. subtilis* minicells either have functional RNA polymerase, unlike *E. coli* minicells, or, like *E. coli* minicells, have the β and β' subunits of RNA polymerase in which case the SP01 phage may inject its own sigma factor along with the DNA to promote initiation of RNA synthesis.

VI. Conjugation Utilizing *E. coli* Minicells

A. Introduction

The decision to utilize minicells in conjugation experiments was based on the facts that the minicell-producing strain of *E. coli* K-12 isolated by ADLER et al. (1966, 1967) was an F^- strain and that minicells could be readily separated from normal sized donor strains of *E. coli*. This last fact made it possible to interrupt conjugation after varying lengths of time with separation of minicells from donor cells under conditions that would preclude alterations in macromolecules in both donor cells and recipient minicells (i.e. low temperature and non-growth conditions).

B. Minicells as Recipients

1. Nature of DNA Transferred to Minicells

COHEN et al. (1967) demonstrated that a variety of donor strains would form specific pairs with minicells derived from an F^- minicell-producing strain. Fig. 7 is an electron photomicrograph of such specific pairs taken by D. P. ALLISON. The general procedure in these experiments was to use donor strains whose DNA had been labeled with [^{3}H]thymidine, to mate them with recipient minicells for varying periods of time, and then to isolate the minicells and determine the amount and type of DNA they contained. It was shown that all donor strains (F^+, F' or Hfr) transferred increasing amounts of acid-insoluble, labeled DNA to minicells as a function of mating time. These studies were reported more fully by COHEN et al. (1968a, b). It was found that this label transfer required that the donor cell be an F^+, F' or Hfr, since no label was transferred to recipient minicells when the labeled parent cell was an F^- strain. Furthermore, there was no transfer when minicells derived from an F^+ minicell producer were used as recipients and transfer was also uninhibited by the presence of deoxyribonuclease during the mating. Thus, the type of transfer observed was seen to possess all of the properties of a true bacterial conjugation system. In addition, electron-microscopic autoradiographic analy-

Fig. 7. Mating between one *E. coli* donor cell and three recipient minicells. Donor pili are demonstrated by lateral attachment of RNA donor-specific bacteriophage. ×19500. Electron micrograph taken by D. P. ALLISON

sis of minicells following matings with labeled donor cells revealed that some minicells had received a substantial amount of DNA, as indicated by the number of silver grains over them, whereas other minicells did not receive any labeled DNA. A representative electron-microscopic autoradiograph of mated minicells taken by Drs. Y. NISHIMURA and L. CARO is shown in Fig. 8.

COHEN et al. (1968a, b) found that minicells mated with all types of donor strains contained principally single-stranded DNA. This conclusion was based on the buoyant density of this DNA in neutral and alkaline CsCl gradients, its elution profile as determined by hydroxylapatite chromatography, its sensitivity to exonuclease I, and on its behavior during and after heat denaturation. In these studies it was shown that the DNA transferred by F^+ donors and by donors possessing short F′ plasmids was converted from the single-stranded state to partially double-stranded DNA in minicells after mating. On the other hand, DNA transferred by Hfr donors and by donors possessing long F′ plasmids remained single-stranded in the minicells. COHEN et al. (1968b) concluded from these results that conjugation involved the transfer of single-stranded DNA synthesized in the donor prior to mating and inferred that it might be necessary for an entire F or F′ plasmid to be transferred before any double stranding of its DNA could occur in minicells. It was also demonstrated that nalidixic acid inhibited conjugal transfer by the donor and vegetative DNA synthesis in the donor to an equal extent, depending upon the concentration of nalidixic acid used. When nalidixic acid-resistant donor strains were mated

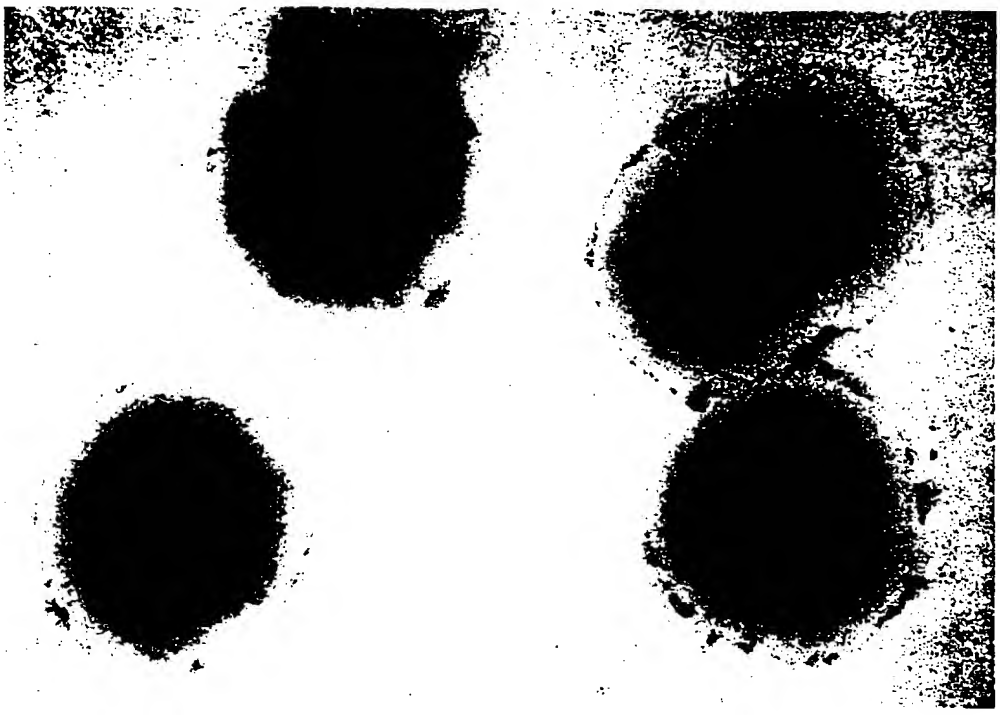

Fig. 8. Electron-microscopic radioautograph of recipient minicells after mating with *E. coli* F+ donor cells whose DNA had been labeled with [^{3}H]thymidine prior to mating. ×30000. Electron micrograph taken by Y. NISHIMURA and L. G. CARO

with minicells, the amounts of transfer and DNA synthesis were unaffected by nalidixic acid. All these results were the same regardless of whether the minicells were derived from nalidixic acid-sensitive or nalidixic acid-resistant minicell-producing strains, and nalidixic acid did not prevent the conversion of single-stranded F DNA to the double-stranded state in minicells. These data corroborated other studies that indicated a dependence of conjugal transfer on DNA synthesis in the donor parent.

COHEN et al. (1968b) observed that the amount of acid-insoluble label transferred from prelabeled donors was about the same regardless of the donor mating type. This suggested that Hfr donors, which are capable of a substantial amount of chromosome transfer to normal sized F$^-$ cells, could not transfer more than a few percent of the length of their chromosome to minicells that were devoid of a chromosome. This finding was in accord with genetic studies (CURTISS et al., 1968), which indicated the necessity for genetic homology between the lead region of the Hfr chromosome and the comparable portion of the recipient chromosome to obtain a significant degree of genetic transfer. In keeping with these results, it was later observed that the weight-average molecular weight of DNA transferred to minicells was usually 20 to 30 × 10^6 daltons independent of the donor strain used (FRALICK, 1970; SHEEHY et al., 1972b; KHACHATOURIANS et al., 1974; GLATZER and CURTISS, unpubl.).

FRALICK (1970) and SHULL et al. (1970, 1971) demonstrated that both Hfr and F DNA conjugally transferred to minicells was attached to the minicell

membrane (60 to 90%) by use of the M-band technique of TREMBLAY et al. (1969). It was therefore evident that the inability of conjugally transferred Hfr DNA to be converted to double-stranded DNA in minicells was not due to the inability of such Hfr DNA to associate with the minicell membrane. As a means to understand the nature of this association between DNA and membrane, SHULL et al. (1971) utilized ionizing radiation to see what percentage of the labeled DNA was released from the membrane by varying doses. They found that although 500 and 1000 krad would reduce the weight-average molecular weight from 25×10^6 daltons to 7×10^6 and 4×10^6 daltons respectively, there was no reduction in the amount of DNA attached to the membrane. They therefore concluded that the DNA must attach at a multitude of sites. The discovery that conjugally transferred DNA associates with the membrane in minicells was in keeping with results obtained by FALKOW et al. (1971) that indicated that conjugally transferred R plasmid DNA initially is associated with the cell membrane in recipient cells. FRALICK (1970) also showed that an exposure of 2000 krad to minicells before mating did not have any effect on either the ability of minicells to act as recipients or the association of conjugally transferred DNA with the membrane.

OHKI and TOMIZAWA (1968) had independently concluded that single-stranded DNA was transferred during bacterial conjugation and, along with RUPP and IHLER (1968), had found that the single strand transferred from the donor that was synthesized prior to mating represented a unique strand of the Watson-Crick helix that entered the recipient with a 5′ terminus. It was still uncertain, however, that the single-stranded DNA isolated in minicells after conjugation represented a unique strand of the F plasmid or of the Hfr chromosome. Therefore, FRALICK (1970) and FRALICK and FISHER (1970) undertook studies to determine whether the single-stranded DNA isolated from minicells after mating was or was not a unique strand of the Watson-Crick helix. In order to accomplish this objective they utilized two Hfr strains which transferred their chromosomes in opposite directions, but in such a manner that both Hfr strains transferred the same genetic segment of the chromosome to minicells. By isolating the DNA conjugally transferred by each of these Hfr strains to minicells, it was possible to determine whether the two DNA's would form heteroduplexes, indicating that complementary DNA strands had been transferred during the two matings. Such heteroduplex molecules were obtained, indicating that one of the Hfr strains transferred the Watson strand whereas the other Hfr transferred the Crick strand for the same chromosomal segment. In control experiments, there were no heteroduplexes formed with DNA transferred by either Hfr alone, indicating that both Hfr strains transferred a unique strand of the Watson-Crick helix.

KHACHATOURIANS et al. (1972, 1974), SHEEHY et al. (1972b), and SHEEHY (1972) studied the degradation of conjugally-transferred single-stranded DNA in minicells. Minicells were purified after 60-min matings with an Hfr donor by two successive sucrose gradients and then examined for loss of acid-insoluble material as a function of time of incubation under a variety of conditions. It was

shown that there was more solubilization of conjugally transferred DNA when minicells from stationary-phase cultures were used in matings than with minicells obtained from log-phase cultures. It was also found that the amount of degradation was stimulated by divalent cations such as Ca^{++}, Mg^{++} and Mn^{++}, in decreasing order of effectiveness, and could be greatly lessened by incubating minicells in an EDTA-Tris buffer. Inhibitors of energy metabolism such as 2,4-dinitrophenol also markedly decreased the amount of degradation. In no instance was more than 40 to 50% of the conjugally transferred DNA degraded. It is difficult to assess the biological relevance of these studies, however, since it is not known whether such degradation does or does not occur during the time of mating or whether its occurrence is a result of subsequent purification of the minicells after mating and of incubation conditions.

In terms of the procedures for using minicells as recipients in conjugation experiments, the results obtained during the past 6 years have given rise to improved levels of DNA transfer. For example: COHEN et al. (1968b) observed that between 0.2 to 0.4% of the total donor label was transferred to minicells, whereas FRALICK (1970) achieved transfer of about 2% of the donor DNA. On the other hand, by conducting matings on membrane filters, SHEEHY et al. (1972b) obtained transfer of about 5% of the donor DNA to minicells during a 60-min mating. The main considerations in achieving these high levels of transfer have been to choose appropriate densities of minicells and donors. In more recent experiments, donor strains have been grown to early-log phase (2 to 3×10^8/ml) in the absence of aeration according to the methods of CURTISS et al. (1969). Recipient minicells have been harvested from either log-phase (SHEEHY et al., 1972b) or stationary-phase cultures (FRALICK, 1970; SHULL et al., 1971; FENWICK and CURTISS, 1973a) by differential centrifugation (COHEN et al., 1968a) and/or by glycerol gradient (SHULL et al., 1971) or sucrose gradient (SHEEHY et al., 1972b; FENWICK and CURTISS, 1973a) centrifugation. Our general impression after using many of these methods is that minicells from log or late-log phase cultures that are purified on linear sucrose in BSG gradients (with or without prior differential centrifugation) act as better recipients than minicells grown and/or purified by other means. Typically, equal volumes of donor cells at an $OD_{620\,nm}$ of about 0.3 (equivalent to about 3×10^8 cells/ml) and minicells adjusted to an $OD_{620\,nm}$ of about 0.4 (about 2×10^9 minicells/ml) are mixed to initiate a mating. Utilizing these procedures, it has been possible to isolate a significant amount of conjugally transferred DNA from recipient minicells (SHEEHY et al., 1972b; FENWICK and CURTISS, 1973a).

2. Molecular Basis of Entry Exclusion

COHEN et al. (1967, 1968b) had noted that there was a substantial decrease in the amount of DNA transferred from donor strains to minicells produced by an F^+ minicell-producing strain. Since the minicells from such F^+ minicell-producing strains did not possess very many F pili, it was inferred that donor

strains must possess another surface component that either facilitates conjugal pairing with recipient strains or prevents pairing with other donor strains. SHEEHY et al. (1972a, b) reinvestigated this phenomenon, which has been termed entry exclusion. It was shown in these studies that DNA transfer was decreased some 20-fold when the minicell recipients harbored a conjugative plasmid that was homologous to the plasmid possessed by the donor strain. In examining the DNA that was transferred under excluding conditions, it was found that this DNA had the same number-average and weight-average molecular weight as the DNA transferred to F^- minicells. It was also shown that the DNA transferred under both excluding and non-excluding conditions was degraded at the same slow rate. It was therefore concluded that entry exclusion was a phenotypic property that was not expressed by 100% of the "recipient" minicells but that, when it was expressed, there was absolutely no DNA transfer. This led to the inference that entry exclusion prevents pair formation between donor cells, a result that had been inferred from other studies utilizing transfer-defective mutants of F (ACHTMAN et al., 1971).

3. Mechanism of Conjugal Replication and Transfer

Minicells have also been utilized to investigate the macromolecular events occurring in the donor cell during conjugation. CURTISS et al. (1970) utilized F^- minicells produced by a minicell-producing strain with the *dnaB* (TS) mutation that blocks DNA replication at 42° C in matings with *dnaB*(TS) donor strains in preliminary experiments to determine the role of DNA synthesis during conjugation. By choice of isotopic label, it was possible to demonstrate that the amount of conjugal DNA synthesis occurring in the donor was equal to the amount of DNA physically transferred to minicells. These results also indicated that vegetative chromosome replication was independent of conjugal replication in the donor, as originally proposed by JACOB et al. (1963). It was also shown that the *dnaB*(TS) minicells were able to convert single-stranded F DNA to double-stranded F DNA at 42° C, which indicated that the DNA polymerases responsible for this activity were not thermolabile in the *dnaB*(TS) mutant.

FENWICK and CURTISS (1972), FENWICK (1973) and FENWICK and CURTISS (1973a, b, c) also utilized recipient minicells to elucidate in greater detail the molecular events occurring in the donor parent during the initiation of conjugal transfer and replication. They utilized a donor strain possessing the *dnaB*(TS) mutation and the plasmid R64-11. Matings were conducted at 42° C under conditions that did not permit vegetative replication of the plasmid or of the chromosome in the donor strain. When [^{3}H]thymidine was added at the initiation of mating, it was shown that F^- minicells, but not R64-11-containing minicells, stimulated a specific type of DNA synthesis in the donor parent and that this occurred in DNA molecules that could be isolated as CCC plasmid

DNA molecules. Thus, under conditions in which entry exclusion was operative, there was no initiation of conjugal DNA synthesis in the donor parent or any evidence for DNA transfer to minicells. By utilizing prelabeled donor strains, FENWICK and CURTISS (1973a) found that maximum transfer of labeled plasmid DNA to minicells was achieved within 20 min. Plasmid DNA isolated in recipient minicells was found to exist as single-stranded DNA with a molecular weight equal to the molecular weight of a single strand of the R64-11 plasmid. No evidence was obtained for continuous R plasmid transfer to give rise to molecules of lengths greater than the length of a single DNA strand of R64-11. In preliminary experiments (FENWICK, unpubl.), it was observed that the conjugal replication of the plasmid DNA in the donor was occurring while the plasmid DNA was associated with the donor cell membrane.

In order to determine the roles for protein, RNA and DNA synthesis during the initiation and continuation of conjugal replication and transfer, FENWICK and CURTISS (1973b, c) employed chloramphenicol or rifampin, and nalidixic acid, respectively. In these experiments it was observed that vegetatively growing donor cells possessed all proteins necessary to initiate one round of plasmid transfer in the absence of further protein synthesis. However, the presence of chloramphenicol during the mating prevented the initiation of a second round of transfer and caused the accumulation of CCC DNA molecules (FENWICK and CURTISS, 1973b). On the other hand, the addition of rifampin prior to the commencement of mating blocked the initiation of conjugal replication and transfer. It was inferred from these studies that donor cells had to synthesize a type of RNA molecule that: (a) was presumably used as a primer for initiation of conjugal DNA replication; (b) was not present in vegetatively growing donor cells; and (c) was not translated into a protein product (FENWICK and CURTISS, 1973b). The studies with chloramphenicol and rifampin gave further support to the conclusion that no more than one genome equivalent of R64-11 DNA was transferred at a time and that transfer required the synthesis and activity of both specific plasmid-specified proteins and of a non-translated RNA to initiate each round of plasmid conjugal replication and transfer. In experiments utilizing nalidixic acid to block DNA synthesis (FENWICK and CURTISS, 1973c), it was observed that the addition of low concentrations of nalidixic acid (10 μg/ml) to the donor cells and recipient minicells prior to commencement of mating caused an equal reduction in both conjugal DNA replication and in conjugal DNA transfer. Nalidixic acid was shown to cause a reduction in the molecular weight of the DNA transferred to minicells, which suggested that this drug might induce single-strand breaks in the plasmid DNA that was being transferred. It was further observed that nalidixic acid caused a nonspecific association of plasmid DNA with the donor cell membrane with a marked reduction in the amount of plasmid DNA isolatable as CCC DNA molecules. These results were interpreted to indicate that nalidixic acid acts by blocking conjugal DNA replication and transfer at the replication fork by some type of nalidixic acid-promoted association between the plasmid DNA and the membrane.

The studies conducted by FENWICK and CURTISS (1937a, b, c) in conjunction with previous elegant work reported by VAPNEK and RUPP (1970, 1971), VAPNEK et al. (1971), and FALKOW et al. (1971) give a rather complete picture of the mechanism of plasmid DNA conjugal transfer. It is evident that plasmid DNA exists during vegetative growth as CCC DNA molecules in the cytoplasm of donor cells during the period when the plasmid DNA is not undergoing vegetative replication. When a suitable recipient is contacted, plasmid DNA associates with the membrane of the donor cell and is converted to a state which permits the transfer of a unique single strand of the plasmid DNA with the 5′ terminus of the single-stranded DNA entering the recipient cell or minicell first. Presumably, plasmid-specified proteins present in vegetatively growing donor cells are used to effect this transition of CCC plasmid DNA to either an open circular or a linear duplex molecule on the donor cell membrane. It should be pointed out that the evidence indicating a discontinuous type of plasmid transfer does not require the existence of open circular plasmid DNA molecules during conjugation as is required by the rolling circle model for continuous conjugal transfer (GILBERT and DRESSLER, 1968). Conjugal DNA synthesis is then initiated by the synthesis of a rifampin-sensitive product (presumably an RNA primer that is not translated into a protein product) and thereafter occurs at a rate which determines the rate of transfer of the unique single plasmid strand and in an amount which is equal to the amount of plasmid DNA transferred to recipient cells or minicells. This newly synthesized DNA replaces the strand transferred and thus ensures the genetic integrity of the plasmid in the donor cell. Upon completion of a single round of plasmid transfer the plasmid DNA dissociates from the cell membrane and reassumes a CCC state in the donor cell. The cycle can then recommence, with the requirement that one or more plasmid-specified proteins needed to initiate the process be synthesized. It should be mentioned that, although it has been assumed that an endonuclease is necessary to initiate a round of plasmid transfer by conversion of CCC DNA to OC DNA, this protein is in fact misnamed, since it is not an enzyme acting catalytically but rather a protein that is consumed in the act of performing its biological function. In the recipient cell, the conjugally-transferred single-stranded plasmid DNA attaches to the membrane and is there converted to a linear duplex molecule, which is converted to an OC and then a CCC form in the cytoplasm.

4. Isolation of Specific DNA Segments in Minicells

FRALICK (1970) and FRALICK and FISHER (1970) utilized recipient minicells to isolate specific DNA sequences transferred to minicells by different Hfr donors. By an appropriate choice of donor strains transferring the same chromosomal segment in opposite directions, they were able to isolate double-stranded heteroduplex DNA that contained a small genetically defined segment of the *E. coli* chromosome.

GLATZER and CURTISS (1972; in prepn) utilized this same system to prove that an Hfr donor transfers part of the F plasmid DNA sequences as the leading extremity of the Hfr chromosome. Isotopically labeled, conjugally transferred, lead-region Hfr DNA was isolated from minicells and was used in hybridization experiments with purified F DNA to show that about 30% of the F sequence is initially transferred by the Hfr. Thus, the site on F involved in integrating F into the bacterial chromosome to yield an Hfr donor is distinct from the site on F for the initiation of chromosome or F transfer.

C. Minicells as Genetic Donors

KASS and YARMOLINSKY (1970) were the first to show that plasmid-containing minicells were capable of acting as genetic donors of the plasmid in matings with normal sized F^- cells. They studied minicells that contained either F' *gal* or F' *gal* (λ) and observed that 15% of the minicells that contained plasmids (only 1% of the total minicells) were capable of acting as genetic donors in a given mating period. LEVY and NORMAN (1970) demonstrated that minicells containing one or the other of two different R plasmids had the same donor ability as the parent cells from which they were derived. ROOZEN et al. (1971 a) likewise demonstrated that F'-containing, R plasmid-containing and Col-*trp*-containing minicells were able to act as genetic donors. In the instances studied by these workers, however, most of the minicells contained F' DNA and it was possible to show that 10 to 20% of the minicells isolated from such F'-containing minicell-producing strains acted as genetic donors of the F' plasmids. Minicells containing F' DNA were able to transfer genetic information contained on the F' but not the chromosomal genetic information transferred by F'-containing normal sized donor cells. This observation provided further evidence that the DNA in minicells from plasmid-containing strains was plasmid DNA rather than chromosomal DNA (KASS and YARMOLINSKY, 1970; ROOZEN et al., 1971 a). Fig. 6 is an electron micrograph taken by D. P. ALLISON of a minicell possessing the F' *lac* plasmid. Note that this minicell possesses two F pili that are specifically marked by their ability to attach donor-specific RNA phages along their length and a donor-specific DNA phage to the tip of one pilus.

As originally noted by KASS and YARMOLINSKY (1970) and confirmed by ROOZEN (1971), (see ROOZEN et al., 1971 b), CCC F' DNA is not isolatable from minicells containing such DNA (less than 1 to 3% of DNA). This is a paradox in view of the ability of these minicells to act as donors of the F' plasmid DNA and the ease with which CCC DNA molecules can be isolated from other types of plasmid-containing minicells (up to 70% of the DNA). These observations could indicate that F' DNA in minicells either is not in the CCC form or that the standard methods for lysing minicells and examining for such DNA convert this DNA to open circular or linear duplex DNA. In any event, the question of the molecular state of F' DNA in minicells is of some interest and is being currently investigated in our laboratory.

VII. Conclusion

The isolation of minicell-producing strains in *E. coli*, *S. typhimurium* and other *Salmonella* species, *B. subtilis*, *H. influenzae*, *Erwinia amylovora*, and marine pseudomonad B-16 implies that such strains could be isolated in a diversity of microbial species. It is evident that the existence of minicells has contributed significantly to our understanding of the molecular biology of bacterial conjugation and of the genetics and molecular biology of plasmids. There is clearly much more that can be done relative to the function and expression of plasmid genetic information using the minicell system. The production of minicells has and should continue to be of importance in studying the organization of the bacterial cell with regard to the partition of enzymes and other cellular constituents. Certainly, the utilization of minicell-producing strains to study membrane and cell wall synthesis should provide information of interest. Such strains should also be useful in understanding the mechanism of cell division as related to the replication and segregation of genetic information in the cell. Minicells should be suitable for "aging" experiments including a search for stable mRNA molecules that may be made by certain microorganisms and for studies on the stability of proteins and of certain biological processes. Although minicells have not been extensively used by investigators interested in passive or active transport, the fact that they lack DNA and the ability to synthesize macromolecular constituents and can be made into protoplasts should make them as useful as red blood cells are to those working with eukaryotic organisms for studies on transport in microbial systems. Lastly, the use of minicells produced by pathogenic species of bacteria may provide a means to develop a live vaccine that will confer long-lived protective immunity, as was suggested by TANKERSLEY (1970) and TANKERSLEY and WOODWARD (1973).

The list of potential uses for minicells and the reasons for isolation of minicell-producing strains in other species is not intended to be all-inclusive but rather suggestive. It is our hope in writing this review that research with these systems will be intensified and that others will be stimulated to think of novel uses for minicells and minicell-producing strains to elucidate some of the more complex biological phenomena that have as yet received too little attention.

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Appendix I. Methods Used to Isolate Minicell-Producing Strains in Different Genera

In most instances the isolation of minicell-producing mutants has been fortuitous, since there seems to be no general selective procedure available for obtaining minicell-producing strains. The usual protocol has been to mutagenize cultures and examine colonies from surviving cells by microscopy to detect those clones that produce minicells. However, the very successful procedure employed by Epps and Idziak (1970) may become the method of choice for selecting and/or inducing minicell-producing mutants.

The minicell-producing strain of *E. amylovora* was discovered by Voros and Goodman (1965) among spontaneous derivatives of a pathogenic, filamentous isolate from nature.

The P678-54 minicell-producing strain of *E. coli* K-12 was obtained by treating a log-phase nutrient broth culture of strain P678 (Jacob and Wollman, 1961) with the mutagen triethylenemelamine (0.5 mg/ml) and selecting survivors that had an unaltered response to UV-irradiation but an increased resistance to ionizing radiation (Adler et al., 1966, 1967). The minicell-producing mutant (P678-54) was thus discovered fortuitously when a culture of this strain was examined by phase-contrast microscopy.

Epps and Idziak (1970) selected *Salmonella* strains resistant to γ-irradiation by 12 repeated exposures to 18 krad (*S. pullorum*) or 20 krad (*S. anatum*, *S. enteritidis*, *S. senftenberg*, *S. typhimurium* ES878, *S. worthington*) and after each irradiation growth in nutrient broth containing 0.3% yeast extract for 24 h at 37° C. After the final cycle of irradiation and growth, cultures were spread on nutrient agar plates containing 0.3% yeast extract and single colony isolates were subsequently compared to the parental strains with respect to morphology and resistance to antibiotics. A very high proportion of the strains subjected to this regimen of repeated irradiations were minicell-producing strains which also tended to form filaments. The typing of minicell-producing mutants in standard biochemical tests showed that they were fairly similar to the parent strains; however, additional cycles of exposure to higher doses of γ-irradiation (25 and 30 krad) resulted in the survival and selection of mutants that varied considerably from parent strains in biochemical tests and the quantity of lipopolysaccharide produced. The effect of this further treatment on morphology was not reported.

The *S. typhimurium* UT13 minicell producer was selected by its resistance to triethylenemelamine on nutrient agar plates. A stationary-phase culture was spread on plates containing 0.1 mg/ml of the mutagen. Colonies were examined for cell morphology by microscopy, and isolates that contained long cells and seemed to contain some minicells were transferred to plates containing 0.4 mg/ml of triethylenemelamine. After five such transfers, one isolate was obtained that was a minicell-producing strain (Tankersley, 1970; Tankersley and Woodward, 1973). An attempt to repeat this procedure to obtain minicell-producing strains from P678 and several other strains of *E. coli* K-12 was not successful (Stallions, unpublished).

VAN ALSTYNE and SIMON (1971) isolated three minicell-producing mutants among strains selected for filament formation following mutagenesis of *B. subtilis* BR77 with 100 μg/ml of N-methyl-N'-nitro-N-nitrosoguanidine by the procedure of ADELBERG et al. (1965). Filament-forming cells were selected by subjecting washed mutagenized cultures to an enrichment procedure which included allowing cultures to grow from a heavy inoculum (i.e. 10%), centrifuging a portion of the cells in a sucrose gradient, and collecting only the heaviest cell fraction to be subsequently used as inoculum for another cycle of growth. Five such cycles of growth and centrifugation were performed before cultures were plated and the resultant colonies screened to detect the desired microscopic morphology. REEVE et al. (1973) isolated three mutants with independent mutations causing minicell production in *B. subtilis* 168 by selecting revertants for known auxotrophic markers after spreading overnight cultures on appropriate minimal selective plates on which three small crystals of nitrosoguanidine had been placed. Cells in colonies revertant for auxotrophic markers were subsequently screened by phase-contrast microscopy to find the minicell-producing mutants.

The *H. influenzae* Rd minicell-producing mutant was also selected after mutagenesis with nitrosoguanidine and was discovered fortuitously (SETLOW et al., 1973).

Appendix II. Strain Construction of Minicell-Producers by Genetic Manipulation of Genotype

A. General

Table 4 contains a list of minicell-producing strains of *E. coli*, *S. typhimurium* UT13, *B. subtilis* and *H. influenzae*. This list is not all-inclusive but it provides information on some of the more important minicell-producing strains of *E. coli* that have unique genetic properties. Many of these strains have not been previously described. The construction of these strains has made use of conventional methods for the isolation of specific mutant derivatives, and of generalized transduction and conjugation for introducing specific mutations that alter recombination, repair, DNA synthesis, cell division, the presence of nucleases, etc. The basic methods for utilizing these techniques in strain construction are given by MILLER (1972).

B. Genetic Preparation of *E. coli* Strains with Different Chromosomal Markers

As indicated previously (Sect. II. C.), *E. coli* P678 from which the P678-54 minicell-producing strain of *E. coli* was isolated (ADLER et al., 1967) possesses at least five mutational lesions affecting galactose utilization (several being suppressor mutations) (BACHMANN, 1972) and probably contains some chromosome rearrangements (ROOZEN and CURTISS, unpubl.). In addition, the fact that two mutational lesions are concerned with the expression of the minicell-

Table 4. Minicell-producing strains

Genus and Species	Strain designation	Genotype[a]	Reference
B. sublilis	VA51[b]	*thr trpC divA51*	VAN ALSTYNE and SIMON (1971)
	VA356[b]	*thr trpC divA35*	VAN ALSTYNE and SIMON (1971)
	VA27[b]	*thr trpC divA27*	VAN ALSTYNE and SIMON (1971)
	GSY1037-*divIVA1*	*ura recA divIVA1*	REEVE et al. (1973)
	RUB770-*divIVA1*	*leuA divIVA1*	REEVE et al. (1973)
	BR59-*divIVA1*	*trpC2 pheA2 divIVA1*	REEVE et al. (1973)
	BR59-*divIVB1*	*trpC2 ilvC divIVB1*	REEVE et al. (1973)
	M11-*divIVB1*	*ura metB divIVB1*	REEVE et al. (1973)
	CU403-*divIVA1*	*thyA thyB ilvC divIVA1*	REEVE et al. (1973)
	CU403-*divIVB1*	*thyA thyB metB divIVB1*	REEVE et al. (1973)
	CU403-*divIVA1 tag-1*	*thyA thyB ilvC divIVA1 tag-1*	MENDELSON and REEVE (1973)
	CU403-*divIVB1 tag-1*	*thyA thyB metB divIVB1 tag-1*	MENDELSON and REEVE (1973)
E. coli K-12	χ925[c]	F⁻*thr ara leu azi*ʳ *tonA*ʳ *lacY T6*ˢ *minA gal λ⁻ minB str*ʳ *malA xyl mtl thi sup*	Single-colony isolate of P678-54; ADLER et al. (1967)
	χ974[aa]	F⁻*thr ara leu T6*ˢ *minA λ⁻minB str*ʳ *malA xyl mtl dnaB* (TS) *sup*	STALLIONS and CURTISS (1971)
	χ964	F⁺ *T6*ˢ *minA λ⁻minB str*ˢ *sup*(?)	see text
	χ984[bb]	F⁻ *T6*ˢ *minA purE λ⁻pdxC minB his str*ʳ *T3*ʳ *xyl ilv cycA*ʳ *cycB*ʳ *met*	see text
	χ1081[cc]	F⁻ *thr lacY proC T6*ʳ *minA purE λ⁻minB his str*ʳ *T3*ʳ *xyl ilv cycA*ʳ *cycB*ʳ *met*	see text
	χ1298	F⁻ *dapD T6*ˢ *minA purE λ⁻ pdxC minB his str*ʳ *T3*ʳ *xyl ilv cycA*ʳ *cycB*ʳ *met*	see text
	P4121	F⁻ *lac minA minB recA argG mal xyl metB thi*	HORI et al. (1974)
H. influenzae	LB11[a b]	*thy-2 str*ʳ	SETLOW et al. (1973)
S. typhimurium UT13	χ1313	prototroph, lysogenic for 3 phages	TANKERSLEY (1970)

[a] Genotype abbreviations are defined by TAYLOR and TROTTER (1972).

[b] VA strains were independently isolated following mutagenesis of the parent strain, BR77, which has the genotype *thr trpC*.

[c] Strains derived from χ925: nalʳ(χ917); P1*kc* lysogen (χ926); *T6*ʳ (χ962); *rif*ʳ (χ1362); *polA4 xyl*⁺ *metE* (χ1235); *thy* (χ1122) and from this *cys* (χ1140); *thy dra* (χ1119 and χ1120) and from these *met* (χ1151, χ1159, χ1160, χ1170, χ1171), *arg* (χ1172), *proA* (χ1248), *proB* (χ1249), *ilvA* (χ1314), *his* (χ1261), *phe* (χ1161), and *malA*⁺ (χ1178). χ1261 gave rise to a *pro* strain (χ1291) and this to a *T6*ʳ strain (χ1391). χ1161 gave rise to a *endI he*⁺ strain (χ1268). χ1178

producing phenotype made genetic manipulation of this strain somewhat difficult. Although many of the useful minicell-producing strains of *E. coli* are direct descendents of χ925 (a single colony isolate of P678-54, selected for its high yield of minicells and ease of separation of minicells from parental cells, see Appdx III. A.), it was also desirable to introduce the genes conferring ability to produce minicells into other genetic backgrounds in hopes of facilitating further studies on the genetic and biochemical bases of minicell production. Since a description of the procedures used to prepare these other derivative strains has not yet been published, the pertinent information on the derivation of some of these strains is given below.

1. *χ974*

χ974 was isolated as an F⁻ Gal⁺ Lac⁺ Thi⁺ Str^r recombinant that inherited the *dnaB*(TS) mutation from χ909 and *minA minB* from χ925. The original DNA temperature-sensitive mutant CR34 (BONHOEFFER, 1966) was converted to an F⁻ prototrophic, phage-sensitive, streptomycin-sensitive derivative by mating with a series of Hfr strains derived from χ15 (i.e. W1485) and then to F⁺ by mating with χ15 to obtain χ909 (STALLIONS and CURTISS, 1971). Thus, χ974 had some of its chromosome replaced with chromosomal material from the W1485 background.

2. *χ964, χ1411*

χ964 is an F⁺ prototrophic, phage-sensitive, drug-sensitive, *dnaB*⁺ minicell-producing strain obtained by mating χ974 with a *serA* mutant of Hfr OR11 (i.e. χ536, described in BERG and CURTISS, 1967). χ964, therefore, had a substantial amount of chromosomal material introduced from the χ15 (W1485) background, since χ15 was the parent of Hfr OR11. χ1411 is an acridine orange-cured F⁻ derivative of χ964 that gives high yields of easily purifiable minicells.

3. *χ984, χ1081*

χ984 was isolated by a mating between χ964 and a multiply mutant F⁻ strain (χ961) isolated directly from the W1485 genetic background. The particular recombinant was selected as a Lac⁺ Pyr⁺ Str^r recombinant. χ1081 was isolated as a PdxC⁺ PyrC⁺ Trp⁺ Str^r recombinant in a mating between χ964 and χ985

was used to derive *recA thy*⁺ (χ1197) and *recB thy*⁺ (χ1247) strains. χ1159 gave rise to a *dnaA* (TS) *met*⁺ strain (χ1201) and then to a *recA thy*⁺ strain (χ1293). Strains derived in laboratories other than the authors' include a *λcI857* lysogen (KASS and YARMOLINSKY, 1970), *thy* (INSELBURG, 1971), *recA* (INSELBURG, 1973), *bgl*⁺ *lac*⁺ WILSON and FOX, 1971), *leu*⁺ (VALLÉE et al., 1972) and *lon* (ADLER et al., 1969). See also Table 3.

[aa] Strains derived from χ974: *polA4 xyl*⁺ (χ1231).

[bb] Strains derived from χ984: *azi^r* (χ1004); *pdx*⁺ (χ1016); *recA his*⁺ (χ1274); *polA4 ilv*⁺ (χ1228); *rif^r* (χ995) and from this *bug-6 purE*⁺ (χ1259 and χ1260); *ara* (χ1276) and from this *T6^r* (χ1392).

[cc] A *rif^r* mutant (χ1207) was derived from χ1081.

[ab] This strain is slightly UV-sensitive, resistant to novobiocin and viomycin, and derived from the Rd strain (SETLOW et al., 1973).

(a *trp* mutant isolated from χ961). Because of the means used to isolate χ984 and χ1081, they probably have 80–90% of their chromosomes derived from the χ15 (W1485) subline (see BACHMANN, 1972, for the pedigree of this line).

4. *χ1298, χ1299*

χ1298 and χ1299 are diaminopimelic acid-requiring minicell-producing strains derived from χ984 by first isolating an *ara*⁻ mutant from χ984 (χ1276) and then selecting for an Ara⁺ Str^r recombinant in a mating with Hfr KL16 (Low, 1972) that possessed a *dapD* mutation. These are particularly useful strains in that the minicells produced are more easily lysed than are minicells from the χ925 and χ984 backgrounds. Furthermore, purified minicells can be incubated in a growth medium lacking diaminopimelic acid with the result that the low number of contaminating parental cells lyse, thus allowing one to incubate minicells for a long period of time under growth conditions in the absence of overgrowth by contaminating parental cells.

C. Preparation of *E. coli* Strains with Various Plasmids

The preparation of minicell-producing strains of *E. coli* possessing various conjugative plasmids such as F, F′, R, ColV, ColB, Col-*trp*, etc. is readily accomplished by standard conjugation techniques (KASS and YARMOLINSKY, 1970; LEVY and NORMAN, 1970; ROOZEN et al., 1971a). The introduction of non-conjugative plasmids such as ColE1, Clo DF13, Tet, etc. presents greater difficulty. INSELBURG (1970) utilized an Hfr donor to facilitate the conjugal transfer of the ColE1 plasmid into the minicell-producing strain. Although this method has general applicability, the recent development of the calcium chloride-cold shock method for transforming *E. coli* cells with plasmid DNA, as developed by COHEN et al. (1972), provides a direct method for transforming minicell-producing strains with non-conjugative plasmid DNA, as has recently been demonstrated by VAN EMBDEN and COHEN (1973).

The preparation of lysogenic derivatives of minicell-producing strains is straightforward with the exception of those strains containing the plasmid *λdv* that were prepared by GRETE KELLENBERGER-GUJER. The *λdv* (susP⁺) genome was isolated by first infecting KM424.1 (a *recA*⁻ strain containing *λdv*⁺ obtained from DALE KAISER) with ϕ21 *b2 susP red*⁺ and purifying heavy recombinant phages that contained *λdv* as a tandem insertion in the ϕ21 genome by CsCl density-gradient centrifugation. The phage from a single plaque that plated on a Su⁻ host was then used to infect a *recA* Su⁻ strain. This lysate contained 50% heavy tandem insertion-containing phage and 50% light ϕ21 *susP*⁺ phage produced by recombination due to the presence of the *red*⁺ phage gene. Purified heavy phage were then UV-irradiated to 1% survival in hopes that *λdv* would recombine out of the tandem insertion and survive, whereas the rest of the carrier ϕ21 would be killed. χ1197 (a λ-sensitive (MalA⁺) *recA* derivative of χ925) was infected with these UV-irradiated phages at low multiplicity

and after 3 h was challenged with a large excess of *λci* to kill all cells that were not immune to *λ*. One of the survivors designated *χ*1256 was *λ*-immune and *ϕ*21-sensitive, and thus had the properties expected of a *λdv* lysogen.

Appendix III. Techniques for Handling Minicells

A. Selection of Strains that Give High Yields of Minicells Easily Separable from Parent Cells

The expression of the minicell-producing phenotype in newly constructed strains is highly variable in both *E. coli* and *S. typhimurium*. Since different minicell-producing clones vary with respect to the uniformity of size of minicells produced, the number of minicells produced, and the size and length of the parental cells, it is important to carefully select the clone to be used in any study. We find it useful initially to screen clones with the phase-contrast microscope to select derivatives that have uniformly small minicells of average diameter about 0.7 μm. We have also found it beneficial to select derivatives that have rather long parental cells, since this facilitates purification of minicells. (It should be noted that this willful selection of minicell-producing strains with large parental cells can and probably has erroneously led some investigators to infer that the mutations responsible for minicell production in *E. coli* also cause cells that are longer than cells in non-minicell-producing strains.) Following initial screening by microscopy, it is a good practice to concentrate the cells and minicells from about 2 ml of culture in 0.2 ml of buffered saline with gelatin (i.e. BSG, CURTISS, 1965) and layer this on a 5-ml linear 5 to 20% sucrose in BSG gradient to determine quantitatively which clone yields the highest number of minicells that are most easily separable from the parent cells. Numerous such gradients can be conveniently centrifuged at top speed in a clinical centrifuge for 20 to 45 min, depending on the centrifuge model. When these gradients are pumped off from the top (Buchler Densi Flow Pump), the OD profile can be monitored with a flow cell and the degree of cell contamination in the minicell band can subsequently be measured. A good minicell-producing strain will give no more than one contaminating parental cell for every 10^4 to 10^5 minicells following a first sucrose gradient centrifugation. It is also prudent to examine both log-phase and stationary-phase cultures, since minicell yields and purity may differ markedly at the two growth stages for a given strain.

In terms of established minicell-producing strains of *E. coli* (i.e. *χ*925, *χ*984, etc.), we have noted gradual decreases in yield and purity of minicells as a function of increasing numbers of strain transfers on slants. It has therefore been necessary periodically to reisolate "good" minicell-producers by the methods described above. If this is not done, one often finds increasing amounts of DNA in preparations of DNA-deficient minicells and high background incorporation of radioactive precursors into DNA, RNA and protein by these minicells. This may well be due to the inclusion in the minicell preparation of

small minicell-like cells that do contain some fibrillar DNA structures, as observed in the electron microscope by TUDOR et al. (1969).

B. Purification of Minicells

Many techniques have been used to purify minicells. When highly purified preparations are desired, a combination of purification procedures is often beneficial. It is critical to determine the level of parental cell contamination in purified minicell suspensions by viable count or direct particle counting because the cell contamination can vary considerably from strain to strain and from experiment to experiment, and also depends on the growth stage of the culture. In general, we have found that minicell yields and purity from *E. coli* and *S. typhimurium* are better from late-log, early-stationary, and stationary-phase cultures than from mid-log phase cultures. The number of minicells/ml can be determined by using a Petroff-Hauser counting chamber with a phase-contrast microscope. The addition of a small amount of safranin stain facilitates detecting minicells (LEVY, 1970b). Minicells can also be quantitated by utilizing a Coulter counter with a small-aperture window (13–19 μm).

1. Differential Centrifugation

VOROS and GOODMAN (1965) used centrifugation to separate *E. amylovora* cells from minicells. Differential centrifugation of *E. coli* cultures can be very effective, reducing the number of cells by two orders of magnitude while leaving about 50% of the minicells in the supernate to be subsequently collected (COHEN et al., 1968a; DVORAK et al., 1970; KASS and YARMOLINSKY, 1970; LEVY, 1970b; SHULL et al., 1971; ROOZEN et al., 1971b; SHEEHY et al., 1972b; FRAZER and CURTISS, 1973; KHACHATOURIANS and SAUNDERS, 1973; KOOL et al., 1974; HORI et al., 1974). An advantage of using differential centrifugation to purify minicells is that physiological shocks can be avoided by maintaining the growth temperature during isolation and by using growth medium rather than buffer for suspending minicell pellets. Centrifugation is often performed at 500 to 2000 $\times$ *g* for 5 min; however, the choice of rotors, centrifugal force, and time of centrifugation is best determined empirically for different minicell-producing strains, because differences among strains with respect to cell length and minicell size may require adjustments in differential centrifugation procedures. In addition, it is important to be aware that differential centrifugation of very dense cell suspensions will result in considerable loss of minicells due to cosedimentation. The degree of purification obtained by differential centrifugation is insufficient for most experiments, even though it is a useful preliminary step in recovering minicells from large culture volumes. However, DVORAK et al. (1970) obtained fairly pure minicell preparations after 3 cycles of differential centrifugation.

2. Differential Rate Sedimentation in Sucrose or Glycerol Gradients

Minicell preparations of high purity (1 cell per 10^6 to 10^7 minicells) can be obtained by two cycles of differential rate sedimentation in linear gradients of

glycerol (10 to 30%) (WICKNER et al., 1972) or sucrose (5 to 20% or 10 to 30%) (ADLER et al., 1967; INSELBURG, 1970; ROOZEN et al., 1971a, b; SHULL et al., 1971; KOOL et al., 1972; FRAZER and CURTISS, 1973; SHEEHY et al., 1973a; REEVE et al., 1973; LEVY and MCMURRY, 1974). However, SILVERMAN (1967) and BLACK (1967) obtained highly purified minicell preparations from χ925 (i.e. 1 cell per 10^6 minicells) by using discontinuous glycerol gradients (15 to 40% in 0.067*M* phosphate buffer, pH 6.7). HORI et al. (1974) used discontinuous sucrose gradients (i.e. 10 ml of 20%, 10 ml of 15%, and 10 ml of 10% sucrose in BSG, CURTISS, 1965) coupled with differential centrifugation to obtain highly purified minicells (1 cell per 10^8 minicells). It should be kept in mind that the concentration of sucrose or glycerol in the gradient as well as the centrifuge and type of rotor will affect the time of centrifugation necessary to obtain separation and may be important in the resultant physiology of the purified minicells, e.g. plasmolysis could be more pronounced for minicells exposed to higher sucrose concentrations. Gradients of sucrose or glycerol should be prepared in buffer and we have found that minicells, at least for conjugation experiments, are more physiologically active when the buffer in the gradients is BSG rather than Tris or 0.067 *M* phosphate buffer.

In purifying minicells on linear 5 to 20% (w/v) sucrose in BSG gradients, we (ROOZEN et al., 1971b; FRAZER and CURTIS, 1973; SHEEHY et al., 1973a) concentrate cultures containing cells and minicells 40-fold (log phase) to 10-fold (stationary phase) in BSG and layer the suspension onto the surface of a 35-ml gradient with a Pasteur pipette in a volume equal to 4 to 8% of the gradient, depending on the density of the suspension. Minicells partially purified by differential centrifugation are concentrated 200-fold (log phase) to 50-fold (stationary phase) before layering on the gradient. Concentrated suspensions should always be vortexed vigorously for about 2 min just prior to layering on gradients to eliminate aggregates and to thus reduce loss of minicells by co-sedimentation with cells and to increase the purity of minicell suspensions. After centrifugation, the minicell band can be conveniently withdrawn from the top of the gradient by means of a syringe the needle of which has been bent at a right angle; for gradients of 35 ml, a 10-ml syringe is adequate. The minicell suspension is then slowly diluted with an equal volume of BSG before centrifuging to pellet the minicells. This diluting procedure may subject minicells to osmotic shock and thus it is best to add diluent slowly in order, if possible, to minimize the osmotic shock. If there is some difficulty in obtaining highly purified minicell preparations, it may be useful to incubate the minicells obtained after the first gradient centrifugation in growth medium, thereby allowing contaminating cells to grow somewhat larger before centrifuging on a second gradient.

For large-scale purification of minicells, FRALICK et al. (1969) utilized 10 to 30% sucrose gradients in the AXII zonal rotor, which is made of plexiglass. Hence, the separation of minicells from cells could be observed directly. Presumably, other zonal rotors can also be used to purify large batches of minicells.

Sucrose treatment is somewhat deleterious to cells, e.g. *B. subtilis* cells undergo considerable autolysis after sucrose-gradient centrifugation and minicells also autolyze to some extent (REEVE et al., 1973). *E. coli* cells have impaired capacity to repair UV- and γ-irradiation-induced damage to DNA following sucrose treatment while minicell capacity for repair was unaffected by sucrose treatment (PATERSON and ROOZEN, 1972b). Cells recovered after sucrose-gradient centrifugation exhibit a delay in initiating growth compared to untreated cells (LEVY, 1970b) and impaired synthetic capabilities have been demonstrated for *E. coli* cells plasmolyzed in 20% sucrose (RUBENSTEIN et al., 1970). LEVY (1970b) has demonstrated that cells subjected to sucrose-gradient centrifugation at 4° C are damaged more than cells centrifuged at room temperature. We have observed significant autolysis of χ925 and χ984 cells after simply chilling cultures on ice, collecting the cells at 4° C, and suspending them in minimal medium at 37° C. Thus cells of χ925 and χ984 are sensitive to chilling as well as to sucrose treatment. However, minicells appear to be more resistant to these treatments because Col-*trp* containing minicells purified at room temperature or 4° C are equally capable of synthesizing anthranilate synthase, while parental cells subjected to this procedure have less synthetic capacity when centrifuged in the cold rather than at room temperature (FRAZER and CURTISS, 1973). This could be due in part to the fact that minicells band at 7 to 10% sucrose and are therefore exposed to a lower sucrose concentration than cells which band or pellet in 20% sucrose in all these gradients.

3. Differential Filtration

Another method of purifying minicells which is based on the difference in cell and minicell sizes is differential filtration. VOROS and GOODMAN (1965) used a Selas porcelain candle filter to separate minicells from cells of *E. amylovora*, and FRAZER and CURTISS (1973) used millipore filters to separate *E. coli* minicells and cells. In the latter procedure, the culture was first subjected to low-speed differential centrifugation to remove most of the cells and the resulting supernate was filtered twice through washed millipore filters of 1.2 μm pore size and once through a filter with 0.8 μm pores. Minicells were then collected by centrifugation of the filtrate. The minicell preparations prepared this way contain about 1 cell per 10^4 minicells. The method obviously is not suitable for isolating minicells from large culture volumes.

4. Decreasing Viable Cells in a Minicell Suspension

Many procedures that selectively kill cells but leave minicells intact have been used either alone or in combination with centrifugation procedures to obtain more highly purified minicell preparations. It should be noted that those methods that cause cells to lyse and liberate cellular constituents that can be taken up by minicells may not be suitable for certain experiments. Likewise, those methods that require long incubations of minicell preparations

during the purification procedure may cause minicells to age with regard to their capacity to carry out various physiological activities.

a) Thermal Induction of Prophage

KASS and YARMOLINSKY (1970) used an *E. coli* minicell producer lysogenized with *λcI857* which produces a temperature-sensitive repressor. Cultures were grown at 34° C and then shifted to 41° C for 70 min to allow thermal induction of λ to occur. Minicells were then purified by differential centrifugation and sucrose gradient centrifugation.

b) UV-irradiation

TANKERSLEY (1970) and TANKERSLEY and WOODWARD (1973) employed UV-irradiation to kill contaminating cells in minicell preparations from *S. typhimurium* that had first been purified by differential centrifugation followed by two sucrose gradient centrifugations. The viable cell to minicell ratio was lowered from $1:10^5$ to $5:10^9$ by this treatment. The success of this method may be due to the fact that the *S. typhimurium* UT13 minicell producer contains 3 different prophages, any one of which might be induced by UV-irradiation (SHEEHY et al., 1973a).

c) Sonication

The purification of *B. subtilis* minicells on sucrose gradients results in considerable autolysis (REEVE et al., 1973) and low physiological activity (MENDELSON et al., 1974). However, physiologically active minicells can be purified following disruption of all cells in the culture by sonication (REEVE and MENDELSON, 1973a, b; MENDELSON et al., 1974). Late-log phase cultures are sedimented and the resuspended pellet is then sonicated at 0° C for 7 one-min exposures, with one-min cooling periods between. The minicells and remaining cells are diluted with growth medium, sedimented and then suspended in a small volume of growth medium and sonicated again. After washing, the remaining debris is separated from the minicells by rate sedimentation in capillary tubes in which the lower band corresponds to the purified minicells and the upper band to cell debris.

d) Differential Centrifugation and Sonication of Penicillin-Induced Filaments

KHACHATOURIANS and SAUNDERS (1973) have developed a method for lysing cells by sonication after inducing cells to form filaments. Partially purified minicells were prepared by differential centrifugation and then incubated in fresh growth medium in a low concentration of penicillin G (10 units/ml) for 2 h at 37° C during which time cells formed filaments, since cell division, including minicell formation, was inhibited. Differential centrifugation was then repeated to remove most of the large filamentous cells and the titer of contaminating cells in the supernate could be further lowered by subjecting the minicell suspension to sonication. Plasmid-containing minicells prepared in this

way have one contaminating cell per 10^7 to 10^8 minicells and are active in DNA, RNA and protein synthesis and the propagation of T4. It is still necessary to determine whether the brief sonication of minicells results in shearing of plasmid DNA and whether the DNA, RNA and protein synthesis represents functional macromolecular biosynthesis before this method can have wide applicability.

e) Inhibition of Cell Wall Synthesis with Penicillin or Ampicillin

Penicillin treatment has been used to selectively kill growing cells in a partially purified minicell suspension while leaving the non-growing minicells intact. BLACK (1967) used 200 units of penicillin per ml to prevent growth of contaminating cells during aging studies on minicells of χ925, purified on glycerol gradients, and observed that penicillin G at this concentration had no effect on oxygen uptake by minicells during 70 min of incubation at 37° C. LEVY (1970b) has used penicillin-G treatment to purify *E. coli* minicells. Cultures were subjected to differential centrifugation and partially purified minicells incubated for 30 to 45 min in fresh medium to allow contaminating cells to initiate growth. Penicillin was then added to a concentration of 1500 to 2000 units/ml and after incubation for about 1 h, the preparations contained one cell per 10^4 to 10^5 minicells. Higher levels of purification could be achieved by repeating the cycle. We have also tested the effectiveness of ampicillin as a means of purifying minicells and observed results similar to those of LEVY (1970b); however, the effect of preincubating Col-*trp*$^+$-containing minicells with ampicillin (50 μg/ml) for 70 min at 37° C was observed to inhibit the incorporation of radioactive amino acid into protein by 60% while RNA synthesis was unaffected (ROOZEN et al., 1971b). In contrast, LEVY (1971a) showed that 1000 units of penicillin G had no effect on protein synthesis during the 120 min of the incorporation experiment with minicells containing either R64-11 or R222 (i.e. R100). The discrepancies observed in these two studies may derive in part from the fact that different drugs and different plasmid-containing minicells were used. *B. subtilis* minicells also have been purified in this way by subjecting cultures to differential centrifugation and treating the partially purified minicell suspension with 1000 units of penicillin for 3 h (REEVE et al., 1973).

f) Inhibition of Cell Wall Synthesis in Auxotrophic or Conditional Lethal Mutants

Another method of decreasing cell contamination in purified or partially purified minicell suspensions is to starve a *dap*$^-$ minicell producer of diaminopimelic acid, a required cell wall component in *E. coli*. Growing cells lyse, and a drop in viable count of at least two orders of magnitude has been obtained in this way (FRAZER, unpubl.). Any mutant defective in cell wall synthesis could be utilized in a similar manner, e.g. a minicell-producing strain that is apparently thermosensitive for cell wall synthesis (isolated by WEATHERLY and characterized by MATURIN, unpubl. results) since it forms osmotically sensitive cells at the nonpermissive temperature (i.e. 42° C).

C. Methods of Extracting Minicells

Minicell extracts have been made by a variety of procedures in order to assay enzymes, to display RNA and protein species on polyacrylamide disc-gel electrophoresis and to analyze the forms and subcellular distribution of DNA recovered from minicells. Most of the methods discussed in this section have been used only with *E. coli* minicells, except as noted otherwise.

1. Sonication

Minicells are more resistant to sonic disruption than cells; however, satisfactory extracts of minicells have been prepared by a number of workers using this method and exercising great care not to overheat during sonication. DVORAK et al. (1970) extracted minicells suspended in 0.15 *M* NaCl by sonication for 30 sec pulses, in the cold, for a total of 3 min. SILVERMAN (1967) similarly extracted minicells suspended in 0.067 *M* phosphate buffer (pH 6.7) by sonication at 20000 cycles/second (Bronson Sonifier S-100) for 30-sec pulses, in the cold, but found that minicell suspensions had to be sonicated for a total of 5 min, while cell suspensions required sonication for only 2 min. LEVY (1971a) extracted minicells suspended in 0.05 *M* Tris- 1 m*M* EDTA buffer, pH 8, by sonication. Sonicated minicell extracts have generally been used for enzyme assays; however, such extracts have also been used to examine proteins by polyacrylamide disc gel electrophoresis (LEVY, 1971a, 1973).

Recently, LEVY and MCMURRY (1974) have used a sonication procedure to extract minicells prior to differential centrifugation in order to separate soluble and particulate subcellular fractions for subsequent examination of the component proteins by SDS–polyacrylamide gel electrophoresis. Minicells (4×10^8 per ml) in 0.05 *M* Tris buffer, pH 8, were treated with 0.7 m*M* EDTA and 100 μg lysozyme/ml at room temperature for 20 min. Inhibitors of proteolytic enzymes (tosyl-L-phenyl-alanylchlormethane and tosyl-L-lysylchlormethane) were added to a concentration of 0.1 m*M* and the suspension was sonicated for 30-sec pulses to a total of 3 min (LEVY and MCMURRY, 1974).

2. Rupture by French Pressure Cell, Lysis from Without, Toluenization and Grinding with Quartz Sand

Other methods of extracting or lysing minicells for assays of enzymes and other cellular components include rupture by passage through a French pressure cell (SCANDLYN, 1968; FRALICK et al., 1969), toluenization (FRAZER and CURTISS, 1973), grinding with quartz glass (HORI et al., 1974), and lysis from without with bacteriophage (CURTISS, unpubl.). Minicell suspensions must be passed through a French pressure cell under 16000 to 17000 psi to obtain rupture, while 12000 psi is adequate to rupture cells (FRAZER, unpubl.). Toluene treatment can be used to assay β-galactosidase and anthranilate synthase in minicell suspensions. One drop of toluene is added to 1 to 2×10^{10} minicells in 1 ml of 0.1 *M* phosphate buffer, pH 7, and shaken vigorously at 37° C for 20 min (FRAZER and CURTISS, 1973). HORI et al. (1974) used the method

(described by MUTO (1968) for cells) of extracting minicells with 0.01 *M* Tris–0.1 m*M* Mg acetate (pH 7.8) after grinding with quartz sand in order to demonstrate the presence of nascent ribosomal RNA in chloramphenicol-like particles synthesized by F'14-containing minicells. Lysis of minicells from without can be achieved by treating a suspension with UV-inactivated T4 or T6 bacteriophage or with T4 or T6 ghosts at a multiplicity of infection of 10 to 20 (CURTISS, unpubl.).

3. Detergent Lysis Prior to Electrophoresis of RNA and Proteins in Polyacrylamide Gels

RNA profiles from minicells have been obtained by polyacrylamide disc-gel electrophoresis of RNA prepared from extracts of minicells after lysis with 1% SDS in the presence of 2.8% diethyl pyrocarbonate at room temperature in a 0.02 *M* Tris–0.1 *M* NaCl buffer (pH 7.6) followed by phenol extraction and ethanol precipitation (ROOZEN et al., 1971b). KOOL et al. (1974) have utilized a procedure for isolating and characterizing pulse-labeled RNA from minicells which involves lysing a concentrated suspension of minicells (about 2×10^{10} minicells/ml) in a 0.06 *M* Tris–0.05 *M* EDTA buffer (pH 7.5) containing 3 mg proteinase K/ml by adding SDS (to a concentration of 0.43%) and macaloid (i.e. purified bentonite) (to a concentration of 0.215%) and heating in a 97° C water bath for 3 min.

Minicell extracts prepared by lysozyme treatment (1 mg/ml) and lysis by 1% SDS at 0° C in a 0.01 *M* phosphate-5 m*M* EDTA–0.13 *M* β-mercaptoethanol buffer (pH 7) (KOOL et al., 1972) or by lysozyme treatment (2.5 mg/ml) and lysis with 0.4% SDS in a 0.15 *M* Tris–0.5 m*M* EDTA–0.64 m*M* β-mercaptoethanol–12.5% sucrose buffer (pH 8) (KOOL et al., 1974) have been used to obtain profiles of minicell proteins by SDS polyacrylamide disc-gel electrophoresis. VAN EMBDEN and COHEN (1973) subjected minicells suspended in 0.01 *M* Na phosphate buffer (pH 7.2) to three cycles of rapid freezing and thawing before the addition of SDS (to 0.07%) and β-mercaptoethanol (to 2.1 *M*) and heating at 100° C for 1 min to obtain lysates which were subsequently dialyzed overnight at 4° C before SDS polyacrylamide disc gel electrophoresis of proteins. LEVY and MCMURRY (1974) first precipitated minicells from suspension (in the presence of 50 μg SDS/ml) with 5% trichloroacetic acid. The acid-precipitated pellet of minicell material was then suspended in 0.05 *M* Tris buffer, pH 8, neutralized with NaOH, and heated at 100° C for 2 min after the addition of SDS (final concentration 1%) and β-mercaptoethanol (final concentration 1%). The resulting preparation was used for SDS-polyacrylamide gel electrophoresis.

4. Lysis Procedures Used to Characterize DNA from Minicells

Various lysis procedures have been used to prepare DNA obtained from minicells (either plasmid-containing minicells or minicell recipients after conjugation) for analysis on CsCl, CsCl–ethidium bromide, neutral sucrose, and

alkaline sucrose gradients. Six basic procedures for lysing minicells have been used: lysozyme–sarkosyl; lysozyme–SDS; lysozyme–nonionic detergent; SSC (saline sodium citrate)–sucrose–SDS; lysozyme–NaOH; and Tris (Tris-EDTA-NaCl, pH 9.1)–SDS. A number of modifications of these lysis procedures which have been used by different investigators will be reviewed in this section.

a) Lysozyme–Sarkosyl Lysis

The lysozyme-sarkosyl method of BAZARAL and HELINSKI (1968) has been used most extensively and involves washing cells twice in TES (0.05 *M* Tris–5 m*M* EDTA–0.05 *M* NaCl), incubating for 10 min in one tenth the original culture volume with 10% sucrose in TES buffer (pH 8) containing 1 mg lysozyme/ml and 0.5 mg pancreatic ribonuclease/ml, and lysing with sarkosyl at a final concentration of 0.67% at 0° C. COHEN et al. (1971 a) modified this method slightly to increase the amount of lysate. Lysates were analyzed on CsCl–ethidium bromide and neutral sucrose gradients. INSELBURG (1970) has used the method of BAZARAL and HELINSKI (1968) with and without small modifications, i.e. digestion with pronase after lysis (INSELBURG and FUKE, 1970; INSELBURG, 1971) or digestion with pronase before addition of sarkosyl (INSELBURG and FUKE, 1971; FUKE and INSELBURG, 1972; INSELBURG, 1973). Lysates obtained by this method were analyzed on CsCl–ethidium bromide or neutral sucrose gradients. ROOZEN (1971) and ROOZEN et al. (1971 a) modified the procedure by using twice the concentration of buffer and lysozyme and by changing the detergent to a mixture of 1.3% sarkosyl and 0.1% deoxycholate (final concentrations). Lysates prepared in this way were centrifuged in CsCl–ethidium bromide gradients. To obtain lysis of *S. typhimurium* minicells, SHEEHY et al. (1973 a) used the method of CLEWELL and HELINSKI (1969), which is similar to that of BAZARAL and HELINSKI (1968) and includes modifications in the concentration of Tris and a step of digestion by pronase before addition of sarkosyl. Lysates obtained by this procedure were analyzed on CsCl–ethidium bromide gradients.

FRALICK (1970) and SHULL et al. (1971) used a modification of the M-band technique described by TREMBLAY et al. (1969) to examine membrane association of both plasmid and conjugally transferred DNA in minicells. They used the standard pH 7 TMK buffer (0.01 *M* Tris–0.01 *M* Mg acetate–0.1 *M* KCl) containing 0.25 *M* sucrose to convert minicells to spheroplasts by treatment with 600 to 1000 mg lysozyme/ml for 15 to 30 min at 37° C. Lysis was achieved by layering equal volumes of cold spheroplasts and 0.2% sarkosyl over linear 10 to 45% or 15 to 48% sucrose gradients. In some cases 0.001 *M* EDTA was present during treatment with lysozyme at 4° C, and the Mg acetate was added with the sarkosyl at the time of lysis. LEVY (1971 a) also utilized the M-band technique to examine plasmid-containing minicells. He used a pH 8 0.05 *M* Tris–10% sucrose buffer containing 0.3 m*M* EDTA to incubate minicells with 100 mg lysozyme/ml for 8 min at room temperature. $MgCl_2$ was then added to 0.6 mM to neutralize the EDTA and the spheroplast mixture was chilled to 0° C

on ice. A sample of 0.5 ml was then layered over a 15 to 47% sucrose step gradient and lysis was achieved by adding 0.15 ml of 5% sarkosyl in 0.1 *M* $MgCl_2$.

CORNETT and REEVE (1974) utilized a modification of the LEVNER and COZZARELLI (1972) method to lyse phage SP01-infected *B. subtilis* minicells. Minicells were suspended in a solution containing 20% sucrose, 0.025 *M* NaCN, 0.025 *M* EDTA, 1.25 mg lysozyme/ml and 250 mg chloramphenicol/ml and, after 20 min at 30° C, were lysed by adding sarkosyl to 1% final concentration.

b) Lysozyme–SDS Lysis

COHEN et al. (1968a) devised a lysis procedure that was subsequently used by FRALICK (1970) and SHULL et al. (1971). Minicells suspended in 0.1 *M* Tris–0.05 *M* EDTA–0.2 *M* NaCl (pH 8.0) were treated with 0.3 mg lysozyme/ml for 30 min at 37° C, then with 1 mg pronase/ml for 2 h at 50° C, followed by lysis in 0.67% SDS for 10 min at 50° C. Lysates were centrifuged on neutral and alkaline CsCl gradients. The lysozyme-SDS procedure of FREIFELDER and FREIFELDER (1968) was used to obtain minicell lysates for centrifugation in alkaline sucrose gradients by KASS and YARMOLINSKY (1970). PATERSON and SETLOW (1972) devised a lysis procedure which includes KCN in the treatment with lysozyme. Minicells are suspended in 0.03 *M* Tris–0.02 *M* EDTA–0.01 *M* KCN–800 μg lysozyme/ml at pH 8 and incubated for 15 min at 0° C before addition of SDS to a final concentration of 1% and lysis at 37° C. Lysates were subsequently analyzed on neutral sucrose gradients. A procedure for lysing *S. typhimurium* UT13 minicells has been developed that also employs KCN at the time of lysozyme treatment (SHEEHY et al., 1973a). Minicells are suspended in 0.05 *M* Tris–0.02 *M* EDTA–0.01 *M* KCN–800 μg lysozyme/ml at pH 8 and incubated for 15 min at 0° C before lysis by addition of SDS to a final concentration of 0.67%, also at 0° C. Lysates prepared in this way were analyzed on neutral sucrose gradients. The presence of KCN in the methods of PATERSON and SETLOW (1972) and SHEEHY et al. (1973a) is critical for obtaining good lysis at neutral pH (PATERSON, pers. comm.). Yet another lysozyme–SDS procedure has been developed to obtain lysates for centrifugation in neutral sucrose gradients (ROOZEN, 1971). The pH 7 phosphate washing buffer and pH 9.1 Tris lysis buffer of FREIFELDER et al. (1971) were used. Lysozyme treatment (1 mg/ml) was at 0° C for 10 min before addition of EDTA to 0.05 *M* followed by lysis with 1% SDS at 0° C.

c) Lysozyme–Nonionic Detergent Lysis

Several different methods have been described for lysis of minicells after lysozyme treatment by addition of a nonionic detergent, either Brij-58 or Triton X-100. KASS and YARMOLINSKY (1970) used the method of COZZARELLI et al. (1968), which involves suspension of minicells at pH 8 in 0.05 *M* Tris–25% sucrose buffer and incubating with 1 mg lysozyme/ml and 0.05 *M* EDTA for 10 min at 25° C and then obtaining lysis by the addition of Brij to a final concentration of 0.5% at 25° C. Lysates were analyzed by CsCl–ethidium bromide

gradients. ROOZEN (1971) used the pH 7 phosphate washing buffer and pH 9.1 Tris lysis buffer of FREIFELDER et al. (1971) with lysozyme treatment and EDTA addition at 0° C as described above for SDS lysis, except that lysis was obtained by the addition of 0.5% Triton X100 at 0° C. Lysates were similarly centrifuged on neutral sucrose gradients. KOOL et al. (1972) used the lysozyme-containing sucrose–TES buffer of BAZARAL and HELINSKI (1968) to incubate minicells for 10 min at 4° C before lysing with a final concentration of 0.5% Brij at 4° C. Lysates were centrifuged on CsCl–ethidium bromide followed by neutral sucrose gradients. VELTKAMP et al. (1974) used the CLEWELL and HELINSKI (1969) procedure modified by using 1% Brij-58 for lysis.

d) Saline–Sodium Citrate–Sucrose–SDS Lysis

LEVY (1971a) used a modification of the method of BERNS and THOMAS (1965). Minicells were suspended in 0.15 *M* NaCl–0.015 *M* Na_3 citrate (i.e. SSC) in 27% sucrose and lysed by the addition of SDS to a final concentration of 0.5% and heating for 10 min at 60° C. Lysates were incubated overnight with pronase and centrifuged on CsCl and alkaline sucrose gradients.

e) Lysozyme–NaOH Lysis

FRALICK (1970) and SHULL et al. (1971) determined the single-strand molecular weights of DNA conjugally transferred to minicells by preparing minicell spheroplasts by the method of GODSON and SINSHEIMER (1967) and lysing them directly on top of 5 to 20% alkaline sucrose gradients (pH 13), as described by MCGRATH and WILLIAMS (1966).

f) Tris (pH 9.1)–SDS Lysis

The method of FREIFELDER et al. (1971) involves chilling cells, washing in a buffer of 0.01 *M* KH_2PO_4 (pH 7)–1 m*M* $MgSO_4$–0.1 m*M* $CaCl_2$–0.1 *M* NaCl, then gently suspending in a lysis buffer of 0.02 *M* Tris (pH 9.1)–0.05 *M* NaCl–0.02 *M* EDTA followed by lysis at room temperature in the presence of Dow-Corning antifoam A with the slow addition of alkaline SDS (i.e. 0.8 *M* NaOH–1% SDS) to a final concentration of 0.15 to 0.3% SDS. ROOZEN (1971), PATERSON and ROOZEN (1972b), SHEEHY et al. (1972b) and FENWICK and CURTISS (1973a) have used this method of FREIFELDER et al. (1971) to obtain lysis of minicell suspensions without lysozyme after treatment with Tris pH 9.1 buffer and alkaline SDS. Lysates prepared in this way are always analyzed in alkaline sucrose gradients and generally the gradients are overlaid first with the lysis mixture of alkaline SDS and then with the minicell suspension in Tris pH 9.1 lysis buffer so that minicells are actually lysed on the gradient. SHEEHY et al. (1973a) used a modification of this method to obtain lysis of *S. typhimurium* minicells for centrifugation on alkaline sucrose gradients. In this procedure, no NaCl was present in the pH 7 phosphate washing buffer or in the pH 9.1 Tris lysing buffer and the concentration of $CaCl_2$ in the washing buffer was increased from 0.1 m*M* to 1 m*M*.

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